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EXR NOTES

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visceral %%%leishmaniasis%%%
AUTHOR: Badaro R (Reprint); Benson D; Eulalio M C; Freire M; Cunha S; Netto E M; Pedral-Sampaio D; Madureira C; Burns J M; Houghton R L; David J R; Reed S G
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Human T-cell activation by 14- and 18-kilodalton nuclear proteins of %%%Leishmania%%% infantum
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Characterization of a %%%Leishmania%%% tropica antigen that detects immune responses in Desert Storm viscerotropic %%%leishmaniasis%%% patients
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IL-12 enhances Th1-type responses in human %%%Leishmania%%% donovani infections
AUTHOR: Ghalib Hashim W; Whittle Jacqueline A; Kubin Marek; Hashim Faisal A ; El-Hassan Ahmed M; Grabstein Kenneth H; Trinchieri Giorgio; Reed Steven G (Reprint)
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ABSTRACT: IL-12 is a pluripotent cytokine that interacts with NK and T cells to play a central role in the initiation and maintenance of Th1 responses and IFN-gamma production. Because of the interactive relationship between IL-12 and IFN-gamma response to infectious

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rK39: A Cloned Antigen of *Leishmania chagasi* that Predicts Active Visceral Leishmaniasis

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The diagnosis of visceral leishmaniasis (VL), a serious and often fatal parasitic disease caused by members of the *Leishmania donovani* complex, remains problematic. Current methods rely on clinical criteria, parasite identification in aspirate material, and serology. The latter methods use crude antigen preparations lacking in specificity. A previously described cloned antigen, rK39, of *Leishmania* specific for all members of the *L. donovani* complex (*L. chagasi, L. donovani, L. infantum*) was very useful in the serodiagnosis by ELISA of both human and canine VL. The present study demonstrated that rK39 seroreactivity correlated with active disease. The sera from early or self-healing infected subjects reacted with leishmanial lysate and were generally nonreactive with rK39. These data demonstrate the utility of rK39 in the serodiagnosis of VL and as an indicator of active disease.

Visceral leishmaniasis (VL) is a widely distributed disease with high morbidity and mortality [1]. In areas in which it is endemic, its prevalence has been recorded at 1–10 per 1000 persons yearly [1–3]. The tools available for the diagnosis of VL include visualization of the parasite in bone marrow or splenic aspiration [4], demonstration of specific antibodies in sera of infected subjects [5–11], and isolation of the parasite by in vitro culture or by hamster inoculation [1]. However, none of these procedures is sensitive enough for identifying all infected subjects within the spectrum of leishmanial infection, which ranges from asymptomatic to acute. Current serologic tests using crude antigen preparations are severely limited in terms of both specificity and assay reproducibility [5, 7, 9]. Patients with asymptomatic or subclinical (or both) VL usually have relatively low antibody titers that fall into the gray zone, in which cross-reactivity is very high in serologic tests that use whole parasites (e.g., immunofluorescent antigen test, IFAT) or parasite lysate [10]. In addition, the demonstration of parasites in these patient groups is extremely difficult [10, 11].

Recently we reported the cloning of an antigen gene coding for a repeat antigen (rK39) of *Leishmania chagasi* and evaluation of that antigen in detecting specific antileishmanial antibody in sera from patients with VL [12]. The initial characterization revealed that this rK39 was highly sensitive and specific for VL. Here we report the sensitivity and specificity of this rK39 in comparison with crude leishmanial lysate antigen for the detection of specific antileishmanial antibody and hoped to show that seroreactivity to rK39 is an early surrogate marker for disease progression in VL.

Materials and Methods

Study population. Sera from 2162 persons were tested using a Falcon assay screening test (FAST)–ELISA (Becton Dickinson,

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Mountain View, CA) to detect specific antileishmanial antibodies from the following groups, defined according to the visceral leishmaniasis spectrum: Acute VL was diagnosed when the patient had fever, hepatosplenomegaly, anemia, and leukopenia and when a bone marrow or splenic aspirate revealed leishmanial amastigotes in Giemsa-stained smears [3].

Children with subclinical VL were characterized by mild constitutional symptoms such as malaise, diarrhea, cough, poor work-play tolerance, and intermittent hepatomegaly. These patients typically have specific circulating antileishmanial antibodies, and frequently the bone marrow aspirate direct smear is negative [10]. Liver biopsy may demonstrate histologic abnormalities and leishmanial amastigotes [11]. In general, ~60%–75% of patients with subclinical VL will resolve their illness after a prolonged period (mean, 35 months) without specific therapy. These are grouped in a category named subclinical self-healing patients. The remaining subclinical patients, initially indistinguishable from the self-healing group, will progress to full blown kala-azar syndrome in 5–12 months. These patients are grouped in a category known as subclinical progressing to full blown VL.

Asymptomatic *L. chagasi* infection represents a group of subjects with positive antileishmanial serology, yet who remain asymptomatic. The majority of these patients (80%) have a strong positive delayed hypersensitivity to leishmanial antigens for 1–3 years following seroconversion [3].

Acute VL. During 1987–1993, 135 sera from patients with clinically and parasitologically confirmed VL were collected and stored at −20°C. All sera were obtained before the initiation of specific antimonial therapy.

Subclinical VL. During the last 10 years, >100 cases of subclinical VL patients were identified in the town of Jacobina, Brazil, by an annual epidemiologic survey for case detection. The index sera from 45 patients were selected for this study because the patients had been carefully followed over the previous 10 years. Fifteen of them progressed from subclinical VL to acute VL (subclinical progressing to VL group) within 12 months of serologic evaluation. The remaining 30 subclinical cases self-healed during the follow-up observation period (subclinical self-healing group).

Asymptomatic L. chagasi infections. One hundred sera of asymptomatic seropositive persons were evaluated for this study. None of these persons had developed clinical signs or symptoms of VL over a 10-year period.

Epidemiologic surveys. In total, 1125 human sera from the population surveyed in Jacobina were retested by ELISA using both leishmanial lysate and rK39. From all subjects, we routinely obtained basic demographic data, epidemiologic information about leishmaniasis and other tropical diseases, and physical examination for hepatosplenomegaly.

Other diseases. One hundred sera were analyzed from human clinically and parasitologically confirmed diseases including cutaneous leishmaniasis, Chagas' disease, and schistosomiasis *(Schistosoma mansoni)* plus bacteriologically confirmed tuberculosis and leprosy. All sera were obtained from the Infectious Disease Research Unit at Hospital Universitário Professor Edgard Santos (Bahia, Brazil).

Healthy subjects. One hundred sera from persons living in areas of Bahia where leishmaniasis and Chagas' disease are not endemic were also evaluated.

Canine leishmaniasis. A prospective study of the epidemiology of canine VL was initiated in 1989 in Jacobina [13]. The dogs (300–500/year) have been evaluated annually for the presence of *L. chagasi* infection. During this time, 90 dogs were found to have acute VL, with high serologic titers of antileishmanial antibodies and demonstration of leishmaniae in liver, bone marrow, or splenic aspirates. The sera were collected and stored before the dogs were sacrificed by the Brazilian National Leishmaniasis Control Program.

In December 1992, dogs were serologically surveyed in Monte Gordo, 80 km from the capital city of Salvador (Bahia, Brazil). The survey was done as part of an evaluation of an outbreak of human VL identified during the previous 5 years [14]. In a single day, blood was collected from 467 dogs in this small village. Serologic evaluation was done simultaneously with both antigens (lysate and rK39). All dogs were held at home until the serologic results were known. After the results were available, the serologically positive dogs were sacrificed and negatives were released. From each sacrificed dog, an imprint was prepared from spleen tissue for direct examination for *Leishmania* organisms.

Serology was done using both leishmanial promastigote lysate and rK39. Leishmanial promastigote lysate from a clone of *L. chagasi* (MHOM/Br/82/Ba-2) was prepared as described [15]. rK39 was the 298-amino-acid sequence with a predicted molecular mass of 32.7 kDa and an isoelectric point of 4.4. rK39 contained an additional 6.2 kDa of plasmic fusion sequences that we recently cloned from the *L. chagasi* clone as described [12].

FAST-ELISA. Microassay plates (Probind; Falcon, Becton Dickinson, Mountain View, CA) or the lid with beads were sensitized overnight at 4°C with rK39 (50 ng/well) or promastigote lysate (1–2 μg/bead-well), followed by blocking with PBS containing 1% Tween 20 for 1 h at room temperature. The assays were done as described [13].

Statistical analysis. Confidence intervals (95%) were calculated using the standard normal distribution formula for proportions. The *P* values stated for comparisons regarding sensitivity are two-sided and are derived from the McNemar test.

Results

Figure 1 presents ELISA results obtained with a panel of 425 sera from the study populations. The numbers of sera falling within different ranges of absorbance values are shown according to their reactivities with lysate and rK39. The rK39 antigen gave significantly higher absorbance values between the two antigens tested ($P < .05$). In addition, rK39 had no cross-reactivity with sera from other parasitic diseases, while the crude lysate had a specificity of 68%. Most of the cross-reactive sera were from subjects with cutaneous leishmaniasis or Chagas' disease. False-positive reactions did not occur with sera from healthy controls from areas without endemic leishmaniasis or Chagas' disease with any of the antigens tested.

The sensitivities of the two antigen preparations for detecting specific antileishmanial antibodies were compared (table 1). Both lysate and rK39 were highly sensitive (98% and 99%, respectively) using human or dog VL sera. Seroreactivity to lysate was observed in all infection categories, including sub-

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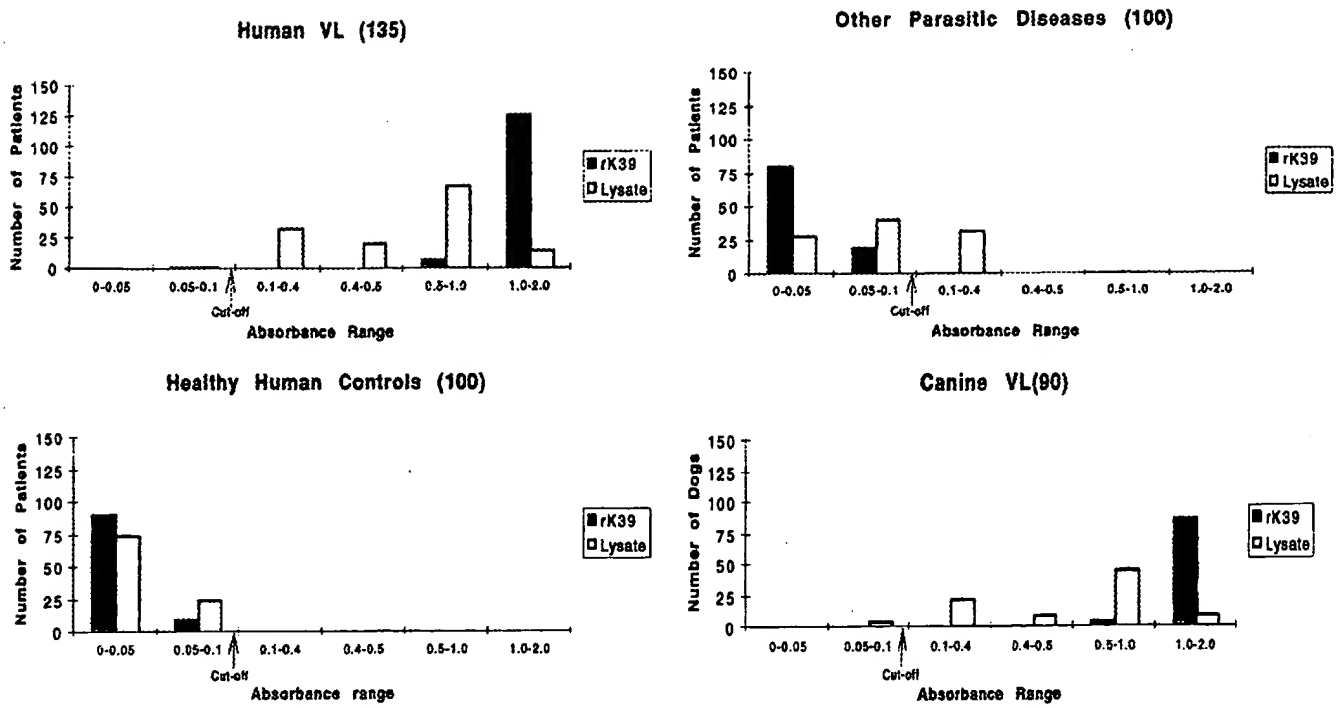

Figure 1. Detection of specific anti–*L. chagasi* antibodies using crude or recombinant leishmanial antigens. Sera from humans (n = 135) or dogs (n = 90) with active VL were compared with control samples from normal subjects (n = 100) or persons with other infectious diseases, as follows: leprosy (n = 20), cutaneous leishmaniasis (n = 20), Chagas' disease (n = 20), tuberculosis (n = 20), or schistosomiasis (n = 20). Cutoff for positivity was determined as described in Materials and Methods. Absorbance values of rK39 recombinant antigen vs. crude lysate for VL sera were significantly different ($P < .05$).

clinical and asymptomatic, as this is a criterion for characterizing leishmanial infection. However, none of the sera from the subclinical self-healing patients and only 4 of 100 sera from asymptomatic children had antibodies to rK39. On the other hand, rK39 detected antibodies in 13 (87%) of 15 index sera from children with subclinical VL who progressed to full-blown disease within 5–10 months.

We also evaluated the specificities of the antigen preparations using sera from healthy controls and from subjects infected with other tropical diseases (figure 1). The rK39 antigen was very specific (100%), with no cross-reactivity among 100 sera from persons with tropical diseases other than VL or 100 healthy subjects. In contrast, crude lysate had high rates of positive reactions with sera from patients with cutaneous leishmaniasis (42%, n = 10), Chagas' disease (50%, n = 20), tuberculosis (30%, n = 20), leprosy (20%, n = 20), and schistosomiasis (20%, n = 20). Therefore, the positive predictive values of a serologic test correlating with human or dog acute VL were as follows: lysate, 64%, and rK39, 98%.

Table 1. Comparison of sensitivity of leishmanial lysate and rK39 to detect antileishmanial antibodies.

Serum category (n)	Lysate	rK39
Human infection, acute VL (135)	98 (96–100)	99 (97–100)
Subclinical progressing to VL (15)	100 (97–100)*	87 (66–100)
Subclinical self-healing (30)	100 (98–100)*	0 (0–2)
Asymptomatic (100)	100 (98–100)*	4 (0–8)
Dog sera, acute VL (90)	94 (89–100)	100 (97–100)
Total (370)	98	65

NOTE. Data are % positive (95% confidence intervals). VL, visceral leishmaniasis.

* Because serologic reactivity with lysate was principal criterion for identifying asymptomatic and subclinical cases, sensitivity is, by definition, 100%.

Epidemiologic survey. To determine the ability of rK39 to detect acute VL, we retrospectively analyzed human sera collected in a cross-sectional survey in the leishmania-endemic area of Jacobina. Among 1125 sera screened with the crude lysate and rK39, 110 (9.8%) were positive with crude lysate, and 11 (1%) were serologically positive with rK39. The rK39-positive sera were among the 110 lysate-positive samples. Follow-up of these 110 persons revealed that, among the 11 rK39-positive sera, 3 were from acute VL and the other 8 were from subclinical cases treated earlier with pentavalent antimony before progressing to the full-blown VL, as we previously recommended [3]. The other 99 lysate-positive sera were from asymptomatic persons who did not develop acute VL.

We also confirmed rK39 seropositivity and presence of parasites in the canine cross-sectional survey. Following the National Visceral Leishmaniasis Control Program, 467 dogs were

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surveyed in a new VL focus (Monte Gordo). Specific antileishmanial antibodies were detected by FAST-ELISA using both lysate and rK39 as the solid phase. Fifty-four dog sera were positive using lysate; of these, 33 were also positive with rK39. These 33 rK39-positive dogs were sacrificed, and leishmania were found in the lymphoid tissues of all of them on direct smear examination.

Discussion

In this study, we confirmed the highly specific nature of rK39 to detect antileishmanial antibodies in acute VL sera (99% sensitivity and 100% specificity). In particular, we discovered that during the acute phase of disease, the host may produce specific antibodies against replicating leishmania, suggested by the observation that sera from patients and dogs with acute VL strongly recognized rK39, but patients with asymptomatic or self-healing infections had low or undetectable levels of anti-rK39 antibodies. On the other hand, in patients with subclinical infections who progressed to full-blown acute VL, antibodies against rK39 were detected a few months before the disease became evident. Therefore, the presence of antibodies to rK39 was 100% correlated with VL. Such a differentiation between asymptomatic and acute VL was not seen using the crude lysate as antigen.

Several methods of serodiagnosis of VL have been used to detect antileishmanial antibodies, including IFAT [5], direct agglutination [6], and ELISA [7, 9]. These tests all use whole promastigotes or lysates thereof. The use of crude parasite preparations for serologic tests presents the problem of cross-reactivity with antibodies from other diseases. Although such conditions may be distinguished clinically, past or subclinical infections with agents of these diseases can complicate diagnosis. In addition, serologic cross-reactivity between leishmania and other infectious agents, such as trypanosomes and mycobacteria [5–8], is a well-documented problem. These problems can be avoided in part by modifications of the assay used, as we have done [7, 15]. However, the best way to increase the specificity of a serologic assay is to use defined antigens. Because high levels of anti-K39 antibodies occur in acute VL, an improved detection system was developed. ELISA wells coated with rK39 have high epitope density compared with lysate-coated wells, resulting in a stronger signal with positive sera.

We have previously described the use of rK39, a potent amastigote antigen shared by members of the *Leishmania donovani* complex [12]. Use of rK39 in serologic assays increases the specificity of the ELISA for VL while retaining sensitivity. The occurrence of rK39 predominantly on amastigotes (the form that replicates and is responsible for pathology) and not in promastigotes (the form transmitted by sand flies) probably explains the high titers of anti-K39 antibody in patients with acute VL and relatively lower titers in those infected but without disease. Such persons, who are without clinical signs or symptoms, have lower numbers of replicating amastigotes in their lymphoid tissue than do patients with acute disease. We believe this to be the most likely reason that rK39 antibodies are not detected in infected persons without disease.

Our data show that rK39 is a serologic indicator of disease from *L. chagasi* infection. Serologic reactivity to rK39 accompanies acute disease and, as we have shown here, also exists in subclinical cases that progress to VL, preceding disease signs or symptoms. Since application of specific chemotherapy before the development of acute symptomatology can significantly improve prognosis, rK39 will prove to be a powerful tool for the management of VL as well as a valuable diagnostic antigen.

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Corporate Source: UNIV IOWA,DEPT INTERNAL MED/IOWA CITY//IA/; VET AFFAIRS MED CTR,/IOWA CITY//IA/52242
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Author(s): TADOKORO CE; MACEDO MS; ABRAHAMSOHN IA
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Abstract: The adjuvant activity of saponin for T-cell responses was evaluated and compared with that of complete Freund's adjuvant (CFA) in two antigen systems: a %%%lysate%%% of the protozoa Trypanosoma cruzi and ovalbumin (OA). Strong delayed-type hypersensitivity and T-cell proliferate responses, comparable with those stimulated by CFA, were observed for both antigens following immunization with saponin as

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Vaccination of Balb/c mice against experimental visceral leishmaniasis with the GP36 glycoprotein antigen of *Leishmania donovani*

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Abstract

Leishmania donovani GP36 glycoprotein is the main antigen of the FML Fucose Mannose Ligand (FML) complex specifically recognized by sera of kala-azar human patients. The GP36 was isolated by chemical elution + sonication and used for Balb/c mouse vaccination in combination with saponin, by the s.c. route, inducing a strong and specific protective effect against experimental visceral leishmaniasis shown by the increase of: specific IgG antibodies (82.6%), mainly IgG2a, the delayed type of hypersensitivity to promastigote lysate (37.8%, $P < 0.001$), the in vitro cellular proliferative response to GP36 of ganglia lymphocytes (53.5%, $P < 0.005$) and the decrease of liver parasite burden (68.1%, $P < 0.025$). Saponin treated controls reacted significantly differently from GP36 vaccinated animals at all the assayed variables ($P < 0.05$). GP36 induced significant protection against murine visceral leishmaniasis at concentrations commonly used for vaccination with recombinant antigens. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Murine visceral leishmaniasis; GP36 glycoprotein; Native antigen; Adjuvants; Saponin

1. Introduction

Human visceral leishmaniasis or kala-azar is a severe disease, lethal if not treated soon after the onset of symptoms, caused by parasites of the *Leishmania donovani* complex. Clinical signs in humans include: malaise, anemia, hepato-splenomegaly, hypergammaglobulinemia, fever, cachexia and progressive suppression of the cellular immune response. About 500000 human cases of kala-azar are registered annually. The disease develops with endemic characteristics in Asia, Europe and America with important localized epidemic bursts. 90% of all human cases occur in Bangladesh, Brazil, India and Sudan. Between 1977–1987, 185000 new cases were registered in Bihar (India). 40000 obits due to the disease took place in Sudan and 400000 annual new cases with 5–7% fatality were reported in India [1]. In America, kala-azar is a canid zoonosis transmitted by sandflies. The control of canine kala-azar should therefore reduce the availability of parasites to sandflies and thus reduce the human disease incidence [2]. The current strategy for control of kala-azar, as recommended by the World Health Organization (WHO), is based on detection and destruction or treatment of infected dogs, treatment of human cases and vector control [2]. Since the efficacy of this control has been shown to be inconsistent, the development of vaccines has been given priority and is considered to be urgent by the World Health Organization.

First and second generation vaccines against leishmaniasis were primarily based on either of two technologies from which antigenic material was derived. These

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were: (1) live attenuated or killed forms of whole organisms: mixed *Leishmania* strains — *L. amazonensis* for humans, in Brazil [3], *L. braziliensis* or *mexicana* with or without BCG in Venezuela [4] and *L. major* with BCG in Iran [5] and (2) defined native or recombinant protein components of the organism obtained by biochemical purification or by genetic engineering. Most second-generation vaccines can be further subdivided into three categories according to their composition: live vaccines, defined subunits and crude fractions. Live vaccines include: attenuated but live parasites [6–8], and recombinant bacteria and viruses carrying *Leishmania* antigens [9,10]. This is the case of the gp63 surface protease gene of *L. major* expressed in *Salmonella typhimurium* [9], and of vaccinia virus expressing the gp46/M-2 gene of *L. amazonensis* [10]. Among the defined subunit vaccines, the gp63 antigen has been studied most extensively [11–13]. The LeIF recombinant analogue of eukaryotic ribosomal protein [14] and the LACK protein or cDNA formulations proved to be protective immunogens for mice [15] while chemically defined antigens of *Leishmania* such as: Lipophosphoglycan (LPG) [16] and Glycoinositolphospholipids (GIPLS) [17] were considered candidates for vaccine formulation. All these antigens, however, demonstrated their protective effect only at Phase I–II levels (safety and immunogenicity, with or without experimental parasitic challenge). Among the crude fractions, the LPGAP, a group of peptides that co-elute with the lipophosphoglycan of *L. donovani* (LPG), were responsible for its previously described immunogenic properties [18]. The DP72, a native glycoprotein of *L. donovani* protected Balb/c mice from experimental visceral and cutaneous leishmaniasis [19]. Finally, the third-generation vaccines are composed of cDNA encoding leishmanial antigens cloned into a eukaryotic expression vector. Preliminary trials with a third-generation vaccine, composed of plasmid DNA containing gp63-cDNA showed that Balb/c mice immunized with this formulation developed significant resistance against cutaneous leishmaniasis [20].

In previous reports, the protective potential of a *L. donovani* promastigote glycoproteic complex was analyzed [21]. This glycoprotein-enriched fraction was named FML ligand, since it contains the neutral sugars fucose, mannose, glucose and galactose and behaved as a ligand that strongly inhibits the in vitro infection of murine macrophages by promastigotes and amastigotes of *L. donovani* [21,22]. This inhibition was species-specific for the genus *Leishmania* [23]. The FML antigen is present on the surface of the parasite throughout the life cycle [22] being a potent immunogen in rabbits and mice [22,24,25] and a sensitive, predictive and specific antigen in serodiagnosis of human [26] and canine kala-azar [27].

Saponins were considered to be the best adjuvants in several different experimental models [28–30] with bacterial adjuvants as a second choice, mainly when cell-mediated immunity is required. They are conjugates of triterpenes, or glycoalkaloids and glycidic moieties, they have been extensively used in vaccination of mice, canine and non-human primate experimental models. Their adjuvant activities were outstanding in vaccines against *Trypanosoma cruzi* [28], cytomegalovirus [29], syncitial respiratory virus [31] and HIV [32]. They are being tested, with WHO support, in large clinical assays against human malaria, melanoma and HIV infection [33].

With the FML antigen of *L. donovani* in combination with Riedel De Haën saponin administered via the intraperitoneal (i.p.) route, an average protection of 87.7% ($P < 0.01$) and 84% ($P < 0.001$) was achieved against visceral leishmaniasis, in the isogenic CB hamster [34] and Balb/c mouse models, respectively [24]. Animals treated with saponin or saline only, showed significantly lower reactivities ($P < 0.01$ and $P < 0.001$, respectively) with no protective effect. No toxic effects were detected at the dosage used [34,24]. No significant differences in antibody response or liver infection were observed among control animals treated only with saline, saponin or FML [34,24]. Protection criteria included maintenance of the delayed type of hypersensitivity, the reduction of splenomegaly [34] and parasitic load, the increase of the in vitro splenocyte proliferation against leishmanial antigen, and the increase in anti-FML specific antibody response [34,24]. The FML was further used in combination with saponin, aluminum hydroxide ($Al(OH)_3$) and Freund's incomplete adjuvant (FIA) in vaccines tested in an outbred murine model of visceral leishmaniasis, either through the i.p. or subcutaneous (s.c.) route [25]. In that work saponin was also the best adjuvant for the FML-vaccine against *L. donovani*, followed by $Al(OH)_3$. The humoral response was significantly higher in the groups treated with FML + saponin or FML + $Al(OH)_3$ than in controls, both before and after infection [25]. Animals immunized by the ip route developed higher antibody titers. A significant and specific reduction of parasitic load in relation to saline (85%, $P < 0.01$) and saponin ($P < 0.025$) controls, was seen in animals treated with FML + saponin by the i.p. route. Coincidental with this reduction, an increase in antibodies of the IgG2a subtype was detected only in animals treated with FML + saponin i.p. A reduction of 88% in parasitic load was achieved by the combination of FML + $Al(OH)_3$ subcutaneously, but the $Al(OH)_3$ treatment itself accounted for 68% of this protection. In our conditions, vaccination with FML + saponin i.p. was superior to other treatments and had no toxic effect due to saponin [25].

FML electrophoretic (SDS-PAGE) analysis disclosed the presence of several proteic bands. Two of them: 36 and 55 kDa, were also stained for carbohydrates [21]. In Western blots, rabbit anti-FML hyperimmune serum reacted with the 36 kDa band [22]. 23 hybridomas secreting IgG and seven hybridomas secreting IgM were cloned. Twenty-two IgG clones recognized the 36 kDa band of FML, and a single clone recognized the 55 kDa band. All the IgM clones recognized only the 55 kDa band. No clone recognized both bands, or other subfractions of the FML. The integrity of the GP36 glycidic moiety was necessary for its antigenic function [22]. No cross reactivity among these two FML fractions was detected. No antigenic homology could be detected among the 36 and 55 kDa bands of FML and the gp63 major surface leishmanial antigen [22]. The 36kDa glycoprotein was identified as the major FML antigenic fraction, a surface glycoprotein antigen of *L. donovani*, and designated 'GP36' [22].

The FML antigen was 100% sensitive and 96% specific in the diagnosis of human kala-azar [26]. In FML–Western blot analysis, the GP36 glycoprotein was the specific marker of human visceral leishmaniasis, labeled only by kala-azar patients' sera. No labeling was detected with sera of patients with tegumentar leishmaniasis, Chagas Disease, or normal controls [35]. Conversely, the 55 kDa component of FML was recognized by normal sera from kala-azar endemic and non-endemic areas, and sera from patients with tegumentar leishmaniasis, kala-azar or Chagas' disease. This antigen is thus non-specific [35].

In the present investigation we pursued the isolation of the GP36 glycoprotein antigen and the analysis of its protective potential in vaccination of Balb/c mice against experimental visceral leishmaniasis.

2. Material and methods

2.1. Isolation of GP36 glycoprotein by chemical elution + sonication

Isolation and chemical characterization of the FML obtained from stationary-growth phase promastigotes of *L. donovani Sudan (LD 1S/MHOM/SD/00-strain 1S)* was performed as previously described [21]. Briefly, promastigotes were submitted to an aqueous extraction followed by heat inactivation and centrifugation. The aqueous supernatant was lyophilized and fractionated by gel filtration on a Bio-Gel P-10 column yielding the FML glycoproteic complex in void volume [21]. For GP36 glycoprotein isolation, the FML antigen was submitted to chemical elution + sonication according to Estalote et al. [36]. The FML was fractionated through SDS-PAGE, under denaturing conditions, in 10% slab baby gels as described [21]. After they were run, gels were stained with Coomassie Brilliant Blue R stain. Sections of the gels corresponding to GP36 glycoprotein were excised and further disrupted into 2–3 mm slices, incubated with 1% SDS, 1% $NaHCO_3$, for 2 h at 22°C with agitation (150 rpm), followed by 16 h at 4°C. The eluted GP36 was submitted to sonication in a Sonifier Cell Disruptor B15 (Branson) (6 cycles × 5 s) and centrifuged at 1180 *g*, 4°C × 30 min. Pellets were discarded and supernatants were dialyzed overnight, against 400 volumes of distilled water. The protein content of GP36 was monitored using a calibration curve relating the SDS-PAGE densitometric profile of known concentrations of BSA to the GP36 sample [36]. Images were acquired using Adobe Photoshop and the NIH Image 1.58 program for densitometric analysis. An anti-GP36 serum was obtained. Briefly, the FML antigen was fractionated by single comb SDS PAGE and the band corresponding to GP36 antigen was excised and homogenized in 0.5 ml PBS (pH 7.2). A three months old rabbit was immunized twice, with a week interval, by footpad injection of 840 μg of GP36 antigen emulsified in an equal volume of Complete Freund's adjuvant (Difco, Detroit, MI) and a third time using the Incomplete adjuvant. Fifteen days after immunization, three intravenous injections of 10 μg of GP36, obtained through chemical elution + sonication, were given at three day intervals. Serum was collected two weeks after the last antigen injection.

2.2. Isolation of GP36 glycoprotein by chemical 'T cell blot'

This was performed according to Kaye et al. [37]. Briefly, FML proteins were applied to a single comb of a 10% SDS-PAGE gel and electrophoretically transferred to a nitrocellulose membrane (0.45 μm; Sigma Co.) in a Western blot apparatus (250 mA × 2 h at 4°C). The region corresponding to GP36 was identified using Rosso Ponceau S (Carlo Erba, Italia) in acetic acid and excised. The nitrocelullose strips (NCP) (5 mm) were dried at room temperature for 12 h, fractionated in small pieces, solubilized with 2 ml dimethylsulfoxide (DMSO) (Sigma Co.) for 2 h at room temperature and precipitated with 5 ml carbonate buffer (pH 9.6). The precipitated NCP were then washed five times in sterile PBS and stored frozen at −20°C [37]. The protein content of GP36 was monitored using a calibration curve that correlates known concentrations of BSA to their SDS-PAGE densitometric profile [36]. Images were acquired using Adobe Photoshop and the NIH Image 1.58 programs for densitometric analysis. Strips containing FML antigen were eluted by the same protocol and used for control of in vitro proliferation experiments.

2.3. Mice

Female outbred Balb/c mice (3-month-old) were obtained from the central animal care facilities, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, RJ, Brazil.

2.4. Immunization and infection of mice

Animals were immunized through the s.c. route, with three weekly doses of GP36 glycoprotein (16 μg) obtained through chemical elution + sonication, and 100 μg of Riedel De Haën saponin (R) in 0.2 ml saline solution. The inoculation was performed in the hind footpads. Saline and adjuvant-treated animals were included as controls. Seven days after immunization, animals were challenged by intravenous injection of 2×10^7 amastigotes (*Leishmania (L.) donovani* LD-1S/MHOM/SD/00-strain 1S), obtained from infected hamster spleens, as previously described [38]. Intradermal reaction against promastigote lysate of *L. donovani* was determined and sera of animals collected, 7 days after last vaccine injection and 15 days after infection. Anti-FML IgM, IgG and IgG subtypes of immunoglobulins were monitored by the FML-ELISA assay. Fifteen days after infection, animals were sacrificed, their *in vitro* ganglia cell proliferation against *L. donovani* antigens was determined and their liver and spleen parasite loads assessed as Leishman–Donovan Units of Stauber on Giemsa-stained imprints (LDU = number of amastigotes/1000 cell nuclei × mg organ weight).

2.5. FML-ELISA assay

The anti-FML antibody levels were assayed in pools of sera collected before and after infection from all the vaccinated groups using the FML-ELISA as previously described [25,26], with 2 μg antigen per well and goat anti-mouse IgG peroxidase conjugate (Sigma) in a 1:1000 dilution in blocking buffer.For the detection of specific antibody types and isotypes against FML (2 μg/well), serial dilutions of immune mouse sera (15 days after infection) were incubated with the antigen, washed and further treated with goat anti-mouse IgM peroxidase conjugate (Sigma) at 1 in 4000 dilution, or with goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 horseradish peroxidase conjugated antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) at 1 in 1000 dilution for 1 h in blocking buffer. The reaction was developed with O-phenyldiamine (Sigma), interrupted with 1 N sulphuric acid, and monitored at 492 nm. Sera were analysed by double-blind tests, in triplicate. Positive and negative control sera were included in each test. All results of serology were expressed as log2 end-point titres. According to conventional serology, titrations differing by two or more dilutions are significant.

2.6. Delayed type hypersensitivity (intradermal reaction to promastigote lysate)

This was determined by injecting mice intradermally, in the right front footpad, with 10^7 freeze-thawed stationary phase promastigotes of *L. donovani* in 0.1 ml sterile saline solution, measuring the footpad thickness with a Mitutoyo apparatus, both before and 0, 24, 48 and 72 h after injection. Controls were performed by injecting each animal with 0.1 ml saline in the left hind footpad. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. Previous experiments carried out in CB hamsters demonstrated that 24 h after inoculation saline treated footpads returned to base levels [34].

2.7. In vitro ganglia cell proliferation against L. donovani antigens

To assess the specific proliferative in vitro response, popliteal ganglia were aseptically removed and disrupted in Hank's saline solution (Sigma Co.) using a Dounce homogenizer. The mononuclear cells were separated by centrifugation at 400 *g* for 5 min at 4°C and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, 0.2g /ml L-glutamine, 0.04 mM 2-mercaptoethanol and antibiotics (200 U/ml of penicillin and 200 μg/ml of streptomycin). The cell suspension was distributed in flat-bottomed microtiter plates (Nunc, Roskilde, Denmark), each well containing 10^6 cells in a final volume of 100 μl. RPMI supplemented medium was added as negative control. 0.4 μg of Concanavalin A, 1.31 μg FML, 0.15 or 0.6 μg of GP36 or 1.25 μg *L. donovani* protein (10^6 freeze and thawed promastigotes) were added to triplicate wells as a stimulus for lymphocyte proliferative responses. Cells were further incubated for periods of 1–5 days at 37°C under a 5% CO_2 atmosphere. Cell proliferation was monitored as described by Mossman [39]. Briefly, 10 μl of a 5 mg/ml solution of [3-(4,5-dimethihyltiazol 2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (Sigma Co.) were added to each well and the plates further incubated for 4 h at 37°C under a CO_2 atmosphere. Reaction was interrupted by the addition of 100 μl 10% SDS in 0.04N HCl. Plates were further incubated at 37°C for 17 h in the dark and absorbancy reading was performed in an Elisa BioRad Microplate Reader Model 550 at 570 nm.

2.8. Statistical analysis

This was performed by a standard *t* test [40].

3. Results

3.1. Isolation of GP36

Fig. 1. shows the GP36 antigen isolated from FML through chemical elution and 'T cell blot'. Isolation by both methods yielded a large and diffuse band in the 36 kDa region (Fig. 1C, 1D). This result is expected for a glycoproteic band. In Fig. 1E, GP36 obtained through chemical elution was treated with an anti-GP36 rabbit monospecific hyperimmune sera. The sharper appearance of the blot band is probably related to the lower concentration of antigen in this nitrocellulose strip (Fig. 1E) and the high antibody titer of the anti-GP36 rabbit serum. A more defined appearence of the band could also correspond to an enhanced reactivity of the serum against proteic epitopes of the molecule. However, this serum showed reactivity against both the carbohydrate and proteic epitopes of GP36 antigen. Indeed, when nitrocellulose strips containing the GP36 antigen were submitted to a mild hydrolysis with 0.1–10 mM sodium *m* periodate in sodium acetate buffer (pH 4.5) and further incubation with 50 mM sodium borohydride solution according to Woodward et al. [41], the carbohydrate epitopes were destroyed and still the anti-GP36 serum labeled the proteic moiety of the antigen. These results were confirmed by an ELISA assay (unpublished results).

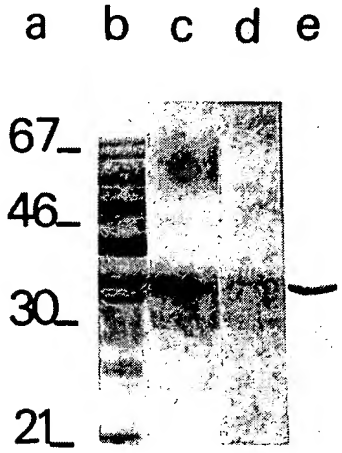

Fig. 1. Polyacrylamide gel electrophoresis of *L. donovani* antigens: lane A, low molecular weight size standards: Bovine Serum Albumin (67 kDa), Ovalbumin (46 kDa), Carbonic Anhydrase (30 kDa) and Trypsin inhibitor (21 kDa) (Amersham, Pharmacia Biotech); lane B, 90 μg of FML antigen; lane C, 0.5 μg GP36 antigen obtained by chemical elution + sonication; lane D, 0.6 μg GP36 glycoprotein obtained through 'T cell blot'; lane E, 0.04 μg GP36 glycoprotein obtained by chemical elution + sonication reacted with rabbit anti-GP36 hyperimune serum (1:1000) and goat-anti rabbit peroxidase conjugate (1:2000). Lane A–D, Coomassie blue stained antigens; lane E, Immunoblot.

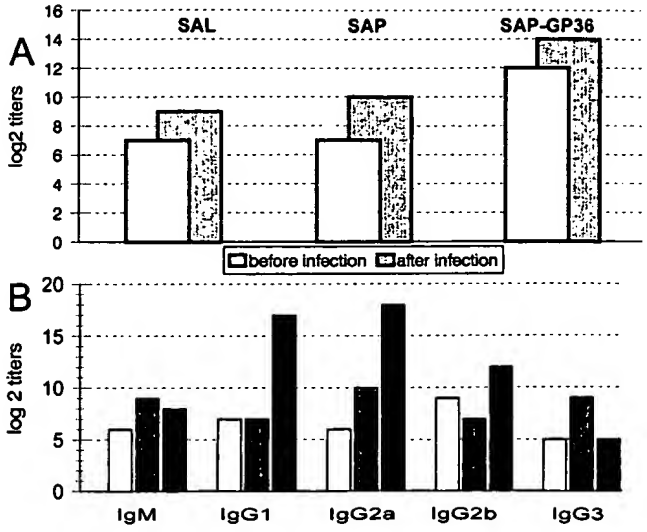

Fig. 2. Anti-FML IgG antibodies in animals vaccinated with the GP36 glycoprotein antigen of *L. donovani* and Riedel de Haën saponin. Balb/c mice were immunized with three doses of 16 μg of GP36 glycoprotein obtained through chemical elution + sonication and 100 μg of saponin (SAP-GP36) by the subcutaneous route (two independent experiments). Control animals received only saline (SAL) or saponin (SAP). The *y* axis represents the $\log_2$ titers of the FML-ELISA absorbance values in the pool of sera obtained from 12 animals for each treatment. (A) Anti-FML IgG immunoglobulins, before and after the challenge. (B) Anti-FML IgM and IgG subtypes of immunoglobulins obtained in sera 15 days after infection. For each set of antibody titers, the vertical bars represent, from left to right, the results of: saline control, saponin and saponin-GP36 treated animals.

3.2. Antibody response in GP36 vaccinated and control mice

Balb/c mice were immunized through the s.c. route, with either three doses of GP36 glycoprotein (16 μg) and 100 μg saponin or saponin or saline as controls. Animals were challenged by intravenous injection of 2×10^7 *L. donovani* amastigotes and their sera was collected, 7 days after last vaccine injection and 15 days after infection. Anti-FML IgM, IgG and IgG subtypes of immunoglobulins were monitored by the FML-ELISA assay. The results represent the mean average of log2 end-point titers for total anti-FML immunoglobulins in pools of sera from each group of animals (two independent experiments, $n = 12$). Fig. 2A shows the anti-FML total antibodies achieved in the immunized animals, either before or after the challenge. The humoral response was specific, e.g. significantly higher in the groups treated with GP36 + saponin than in saponin or saline controls, both before and after infection. Finally, the highest antibody levels were achieved by animals that received the complete vaccine, after the challenge (Fig. 2A).

The IgM and IgG isotypes of specific anti-FML antibodies were measured in all groups of animals,

fifteen days after infection. As seen in Fig. 2B, a significant, although non-specific, increase in IgM antibody titres was observed in GP36 + saponin immunized animals. According to conventional serology, titration differing in two or more dilutions is significant. One desirable feature of adjuvant activity is the capacity to selectively induce the production of protective IgG subclass antibodies that are directed specifically to the antigen. An increase in antibodies of the IgG2a and IgG2b subtypes was detected only in animals treated with GP36 + saponin. Also, a significant and specific enhancement of the IgG1 subclass was observed. This effect was expected as a consequence of the saponin treatment. The IgG3 antibody titers were similar in both the vaccinated and control groups. This immunoglobulin subtype profile composition characterizes a protective response (Fig. 2B).

3.3. Cell mediated immune response in GP36 immunized mice

Cellular immunity against leishmanial parasites was assessed in vaccinated animals by skin-testing with freeze-thawed stationary phase promastigotes of *L. donovani* and by measuring the capacity of ganglion cells from immunized and infected mice to respond to the antigen preparation by in vitro lymphocyte proliferation.

The increase in footpad thickness (IDR) was measured 0, 24 and 48 h after antigen injection. Fig. 3A summarizes the IDR results in vaccinated animals before infection. The swelling was significantly greater in vaccinated animals than in controls, both at 24 and 48 h after antigen injection ($P < 0.005$). Saponin controls were also significanty different from GP36-saponin treated animals, either ($P < 0.005$). These results indicate that the intradermal response corresponds to a delayed type of hypersensitivity considered as a signal of protection or resistence against infection by *L. donovani*. The IDR responses of two independent experiments of vaccination with GP36 and saponin were then evaluated, 48 h after antigen injection. After infection, the thickness increase was significantly greater in animals treated with GP36 and saponin than in those treated with adjuvant ($P < 0.025$) or saline ($P < 0.001$), characterizing a protective response against the infection with *L. donovani*.

Fig. 4 shows the proliferative response of ganglia lymphocytes against GP36 and FML antigens isolated through 'T cell blot'. This evaluation was performed 15 days after challenge. As expected in heavily infected animals a low proliferation is seen against the promastigote protein of *L. donovani*. However, ganglion cells responded to GP36 antigen. Lymphocyte proliferation was significantly greater than control ($P < 0.005$) in cells of GP36 vaccinated animals incubated for 24 h either with 0.15 and for 24 and 48 h with 0.6 μg of GP36. The

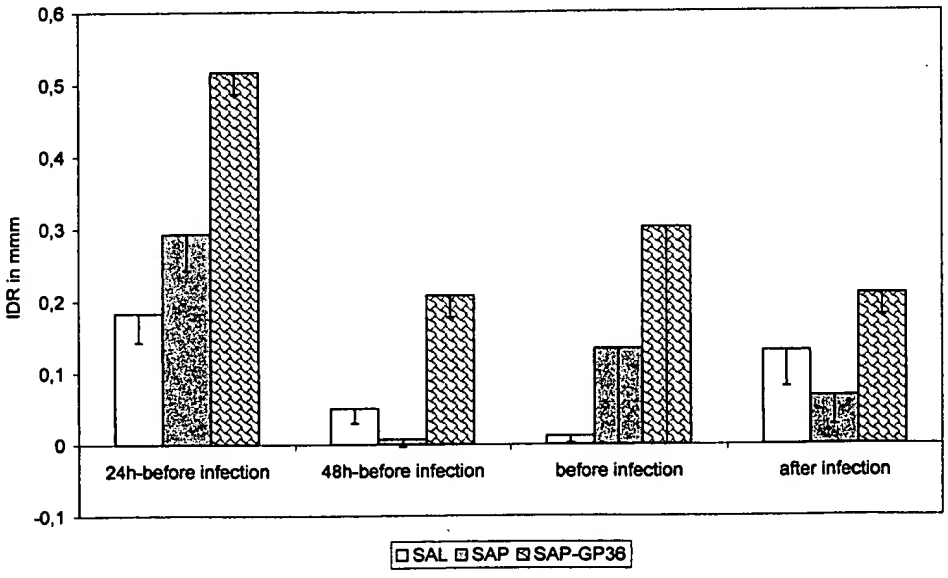

Fig. 3. Delayed type hypersenstivity in Balb/c mice immunized with saponin-GP36. Balb/c mice were immunized with three doses of 16 μg of GP36 glycoprotein obtained through chemical elution + sonication and 100 μg of saponin (SAP-GP36) by the subcutaneous route. Control animals received only saline (SAL) or saponin (SAP). From right to left: the intradermal reaction, before infection, 24 and 48 h after injection with 10^7 freeze-thawed stationary phase promastigotes of *L. donovani*, and the IDR responses of two independent experiments of vaccination with GP36 and saponin ($n = 12$) evaluated at 48 h after antigen injection. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. The *y* axis represents the thickness of skin test in mm. Horizontal bars represent the standard deviation.

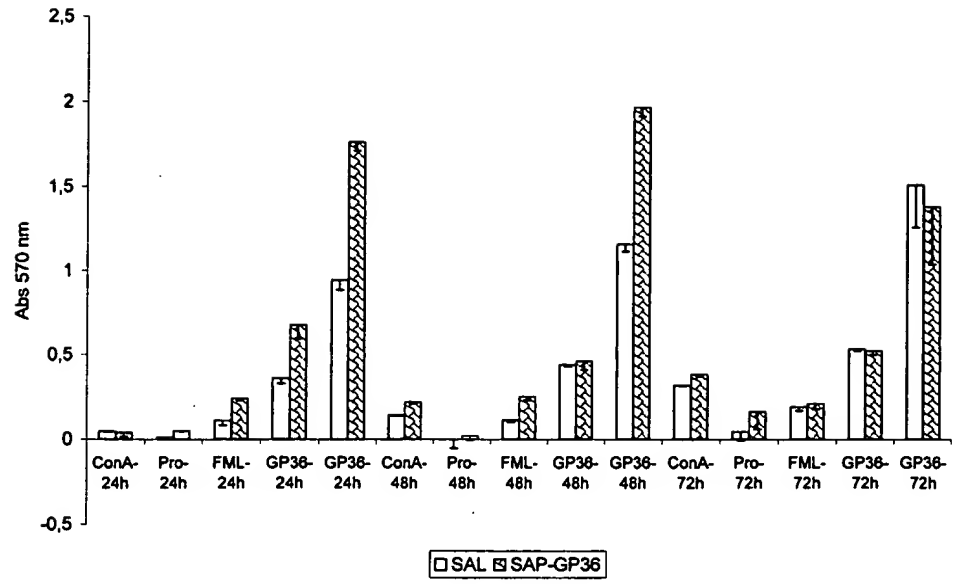

Fig. 4. Proliferative response of ganglia lymphocytes against GP36 antigen isolated through 'T cell blot'. This evaluation was performed in saponin-GP36 vaccinated animals (SAP-GP36) and saline controls (SAL), 15 days after challenge. Triplicates of 10^6 cells, in RPMI supplemented medium, were treated with 0.4 μg of Con A, 1.25 μg *L. donovani* protein (10^6 freeze and thawed promastigotes), 1.31 μg of FML, 0.15 μg and 0.6 μg of GP36 antigen and incubated for 24–72 h at 37°C under a 5% CO_2 atmosphere. Cell proliferation was monitored as described by the MMT method. Horizontal bars represent the standard deviation.The *y* axis represents the absorbency readings at 570 nm.

response was more pronounced against 0.6 μg of the antigen.

3.4. Protection against experimental kala-azar

Previous experiments on the mouse model demonstrated that parasites are evident in liver touch biopsies, 15 days after inoculation with 2×10^7 amastigotes of *L. donovani* [24,25]. The reduction of liver parasitic load in response to GP36 + saponin vaccine is shown in Fig. 5. Animals treated either with saline or saponin developed LDU values close to 1000, while a specific and significant protective effect was achieved in animals vaccinated with GP36 + saponin that showed 68.1% reduction of liver parasitic load. Differences from the saline and saponin control groups were significant ($P < 0.025$ and $P < 0.05$, respectively). Although the Balb/c model is considered susceptible to visceral leishmaniasis, usually this strain does not develop fatal disease [38]. In our investigation, however, 6/20 animals treated with saline and 3/20 treated with saponin died of kala-azar while all animals vaccinated with GP36 + saponin survived until the end of the experiment.

Finally, the survival of vaccinated mice, the reduction of parasite load in the liver, the enhancement of: the anti-*L. donovani* IgG2a and IgG2b specific immunoglobulins, the intradermal reaction to promastigote lysate and the in vitro lymphocyte proliferation against GP36 confirm the protective potential of the GP36 + saponin vaccine formulation against experimental visceral leishmaniasis in the Balb/c mice model.

4. Discussion

4.1. GP36 isolation

The present investigation aimed at the isolation and analysis of the protective potential of GP36 glycoprotein in vaccination against experimental murine visceral leishmaniasis. Previous studies showed that GP36 was a serologically specific marker of human kala-azar [35]. The chemical elution + sonication method, that had proved to be efficient in purification of the Band-3 major antigen from erythrocyte lysate [36], allowed the isolation of the GP36 antigen from FML preparations, showing an apparent molecular weight of 36 kDa (SDS-PAGE) and its use in vaccination of Balb/c mice. On the other hand, and although with a lower yield, the use of the 'T cell blot' method was preferred, in cases of cell proliferation in vitro assays. Different from chemically eluted antigen, no SDS or NaOH contamination was present in these antigen preparations, avoiding any cell toxicity.

The chemical elution + sonication method was also worthy for the GP36 aminoacid and sugar analysis. As previously described in FML antigen [21] and in glycoproteic fractions formerly isolated from *L. donovani* by Olafson et al., [42], a majority of acid and non-polar residues were detected among GP36 components: 13.1% aspartic acid, 11.0% glutamic acid, 7.6% glycine, 10.2% alanine, 7.4% valine and 10.1% leucine. On the other hand, fucose and mannose that were previously characterized in the FML glycidic moiety [21] were disclosed

in GP36 glycidic moiety. The analysis of acetylated residues performed by gas-chromatography disclosed the presence of two different types of fucose residues (2,3-Me_2.Fucose, 2,4-Me_2-Fucose) and a majority of 2,3,6,-Me_3-Mannose units and tri-Me_3-Galactose residues corresponding to short linear chains of 4-*O*-substituted mannopyranose alternating with 3-*O* and 4-*O* substituted fucopyrannose residues (unpublished results). This is the first description of fucose in the genus *Leishmania*. Fucose and mannose were shown to be the more active sugars in competition with promastigotes for the *L. donovani* interiorization receptor on the macrophage surface [21]. The mannose–fucose receptor (MFR) constitutes one of the major routes of entry for promastigotes into murine macrophages [43] and human monocytes [44]. Co-incubation with FML inhibited the promastigote interiorization at very low concentrations (2–6 μg/ml), achieving a 'plateau' of 70–80% inhibition. This kind of curve is typical of specific ligands acting on a saturable number of receptors of the macrophage surface [21]. GP36, then, is an *L. donovani* specific antigen that contains the sugar ligands necessary for macrophage-parasite recognition and penetration.

Cumulating evidence in the literature describes *Leishmania* antigens with m.w. ranging from 32 to 35 kDa recognized by sera raised against membrane preparations of *L. donovani* and *L. chagasi* (reviewed in [22]). Therefore, investigations focused on crude or purified antigens, obtained under denaturating conditions or not, confirmed the existence of a glycoproteic component with apparent m.w. 30–36 kDa in the *L. donovani* complex (reviewed in [22]).

4.2. GP36 immunoprotective potential against experimental murine visceral leishmaniasis

The efficacy of a vaccine formulation relies not only in the specificity of its antigen but also on the ability of the adjuvant to trigger an efficient protective response. For an immunosuppressor intracellular parasite like *L. donovani*, a good adjuvant should guarantee the maintainance of a strong humoral and cellular immune response against the pathogen. In our models with FML-vaccine, the Riedel de Haën saponin gave the best adjuvant performance: the reduction in liver parasitic load of vaccinated animals was 87% ($P < 0.01$) for CB hamsters [34], 84.4% ($P < 0.001$) for Balb/c [24] and 85% ($P < 0.01$) for Swiss Albino mice [25]. Although considered haemolytic in vitro [53], the Riedel de Haën saponin used in combination with FML was not harmful either for hamsters or mice [24,25,34]. Aluminium hydroxide, on the other hand, showed to be haemolytic even when tested at concentrations allowed for use in humans [25]. For all these reasons we choose the Riedel de Haën saponin for the vaccine formulation with the GP36 antigen of *L. donovani*.

In GP36-saponin vaccination experiments the protective effect was evident by the significant and specific increase of: total anti-FML IgG antibodies, intradermal reaction to promastigote lysate, in vitro ganglion cell proliferation and decrease of liver parasitic load

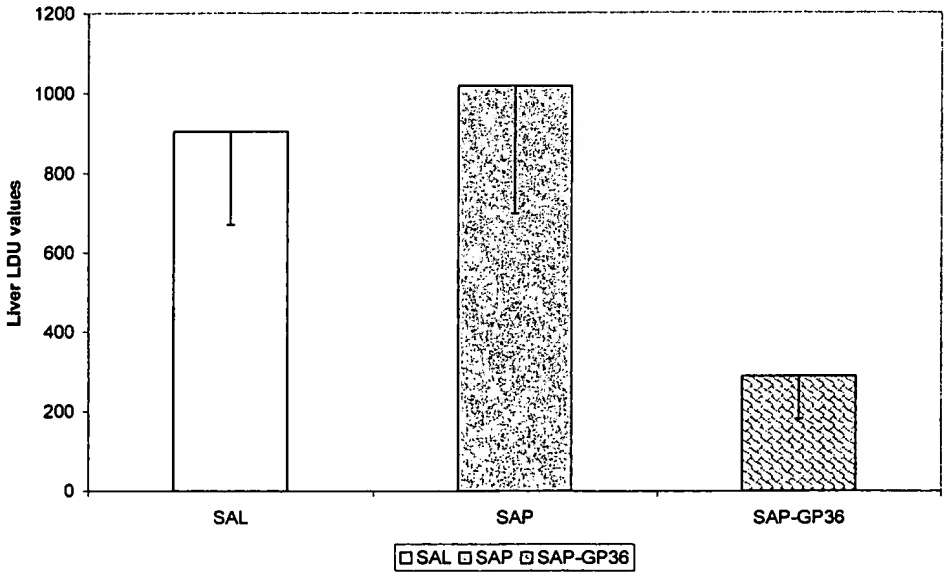

Fig. 5. Liver parasitic burden in saponin-GP36 vaccinated Balb/c mice after infection with *L. donovani*. Animals vaccinated with three doses of 16 μg of GP36 and 100 μg of saponin by the subcutaneous route were challenged with an intravenous injection of 2×10^7 amastigotes. Control animals received only saline (SAL) or saponin (SAP), as indicated. Fifteen days after infection mice were sacrificed and parasites were counted in Giemsa stained liver touch biopsies. Vertical bars represent the mean values of LDU based on parasite counts in 1000 cells for 12 mice in each group. Horizontal bars represent the standard deviation.

(68.1%) when compared to the saline control. Also, a specific increase of anti-FML IgG1, IgG2a and IgG2b antibody subtypes was observed. All these effects were specifically related to the antigen and not to the saponin adjuvant.

If humoral response is related to protection against disease, two aspects should be considered in the interpretation of the success of a vaccination protocol: (1) the quantitative aspect of reaction measured as the total IgG end-point titers and (2) the qualitative protective response, indicated by the increase in IgG2a and/or IgG2b subtypes of immunoglobulins against the candidate antigen. These subtypes are specifically related to a protective response. Regarding the quantitative IgG response: the increase in anti-FML IgG titer after amastigote challenge was of two titer units in GP36 vaccinated animals (12–14), and of three units (7–10) in saponin treated animals. Although a stronger response could be expected of GP36 vaccinated animals, one must consider that, before infection, the IgG antibodies in GP36 treated group, were already significantly higher than controls (12:7:7), and this is one of the desired effects of a vaccination protocol. Furthermore, this anti-GP36 serum specifically recognizes the GP36 band in Western blot analysis against the purified GP36 (Fig. 1E) and the total FML complex (not shown).

On the other hand, the relatively low IgG global response after amastigote infection, could be masking, the protective effect, evident by the IgG2a and IgG2b increase, found only in GP36 vaccinated animals and not in controls. Regarding the analysis of the qualitative antibody response, measured in IgG immunoglobulin subtypes, and considering a difference of two or more titer units as significant, we showed that GP36 vaccinated animals display significantly higher titers than saline controls in IgM (8:6), IgG1 (17:7), IgG2a (18:6) and IgG2b (12:9), while saponin treated animals show increases in IgM (9:6), IgG2a (10:6) and IgG3 (9:5), only. Therefore, although an unspecific increase in the protective IgG2a subtype is seen in saponin treated animals, the difference between this and the titer achieved by GP36 vaccinated animals is highly significant (10:18). These results demonstrate that although diluted in the total IgG global response, the antibody protective response achieved by the GP36 vaccinated animals is strongly significant, specific and correlated to other protection results (IDR increase, and liver LDU decrease).

After challenge, high titers of IgG1 were found in GP36 vaccinated animals (IgG1:IgG2a:IgG2b: 17:18:12). In previous investigation, using the FML antigen and saponin for Swiss Albino vaccination against visceral leishmaniasis, Santos et al. [25] also described high levels of IgG1. Indeed, the ratio of IgG1, IgG2a and IgG2b titers was: 14:12:14, for FML vaccinated group, and 8:8:9 for saponin treated animals. Using the same protocol, the magnitude of the increase in all subtypes was then higher in GP36 vaccinated animals, than in FML vaccinated animals, despite the homologous nature and higher concentration of antigen dosis (150 μg of FML) used in Santos' work [25]. An increase in IgG1 or IgE titers suggests a TH2 response. Our results indicate then a mixed TH1/TH2 response. This kind of profile is the expected for vaccine formulations using saponin. Cumulative evidence in literature have proved that [31,45,46] and although the response is mixed in all systems, saponins induced a highly protective response reducing, parasitemia [25,28,47], viremia [31,45] or bacteremia [46]. Furthermore, saponins are considered promising adjuvants and are now being tested even in human large clinical trials against malaria, HIV and melanoma [33]. The mixed response is also evident by the combined cytokine response (IFN and IL4 or IL10) achieved by mice immunized with antigen and saponin [48,50]. Previous experiments in Balb/c mice vaccinated with GP36 and saponin disclosed a mixed response: supernatants of Concanavalin A in vitro stimulated ganglya lymphocytes of GP36-saponin vaccinated animals secreted 5.624 ng/ml of γIFN and 4.020 ng/ml IL10. No detectable levels of these cytokines were found in supernatants of saponin controls (unpublished results). Recent experiments with FML-QuilA saponin vaccinated Balb/c mice, also showed increased levels of both γIFN and IL10 in animal sera. 93.9 pg/ml of γIFN and 169.4 pg/ml of IL10 were detected in sera of vaccinated animals while 61.3 pg/ml of γIFN and 84.7 of pg/ml IL10 were detected in saline controls ($P < 0.05$). Concomitantly, these animals showed the increase in anti-FML IgG1, IgG2a and IgG2b antibody titers (19:18:18) when compared to saline controls (10:12:12) and the 33% reduction in liver LDU load (Santos et al., unpublished results).

Regarding the cell mediated immune response in GP36 immunized mice, we showed that the in vitro proliferative response was significantly enhanced in cells treated with GP36. The absorbance ratio at 570 nm between SAP-GP36 and SAL-stimulated cultures was 1.88 (0.15 μg–24 h), 1.86 (0.6 μg–24 h) and 1.7 (0.6 μg–48 h). In previous work, with FML saponin vaccinated Balb/c mice, similar ratios (3.0) were achieved [24]. These values, although lower than those generally achieved by thymidine incorporation assays, are the expected from MTT assays [39]. They correspond to the proliferative response of GP36-vaccinees that was significantly higher than that of controls and this is correlated to the protective effect showed by the reduction on liver parasite load and animal survival. No differences between results of colorimetric MTT assays, radioisotope assays or visual inspection of wells for the same system were described [39]. Furthermore, different

from tegumentar leishmaniasis, the supression of the cellular immune response is expected in animals and humans suffering from visceral leishmaniasis. This is seen both, in the in vitro leishmania T-cell proliferation assays [49,50] as well as in the intradermal reaction [50]. Therefore, the achievement of a significant proliferative response and of a positive intradermal reaction after infection with *L. donovani* in GP36-vaccinated animals is worthy.

In this investigation, the IDR reaction reached its maximum at 24 h and was still present 48 h after injection. Even though at 48 h, when the response is expected to be lower, we found significant differences between the vaccinated and unvaccinated heavily infected groups, indicating the protective effect. This behaviour discloses a delayed type of hypersensitivity to the leishmanial antigen and not merely a Jones–Mote response. We previously detected a maximal reactivity 24 h after antigen injection in CB hamsters vaccinated with FML + saponin [34]. In that case, a histological analysis was performed and the microscopic observation of hematoxylin-eosin stained samples showed typical mononuclear cell infiltration.

In this investigation we used 16 μg of GP36 for each vaccination dose. This is a small amount of antigen equivalent to that used in protocols using recombinant antigens [12,51]. Recently, a cystein proteinase, the GP63 glycoprotein and the acid phosphatase of *L. mexicana* were used in combination with IL12, Detox, 4′-monophosphoryl lipid A, QS-21 saponin, BCG and *Corynebacterium parvum*, in mouse vaccination against tegumentar leishmaniasis, achieving different degrees of protection against challenge with promastigotes [12]. All vaccine formulations contained a mixture of the three recombinant antigens, each at a concentration of 2.5 μg/dose [12]. Also, protection against *L. major* infection was obtained with the recombinant LACK protein and rIL-12 [15]. Mice received two doses of 50 μg of recombinant protein and 100 μg of cytokine cDNA. As previously described for the FML antigen vaccine, in this investigation we used Balb/c mice and 100 μg of Riedel de Haën saponin as adjuvant. Immunization with 16 μg GP36 determined a 68.1% ($P < 0.025$) reduction in liver LDU while the use of 150 μg of FML (75 μg of protein) induced a 85% average reduction of parasitic load [24,25]. In vaccination with FML then, a 20% enhancement of protective response was achieved using a five-fold increased concentration of antigen [24,25]. GP36 antigen represents therefore, not only a specific antigenic marker but a very active immunogenic component of the FML complex that might be considered a good candidate for development of vaccine formulations against visceral leishmaniasis.

Using the purified dp 72 native protein an 81–82% of reduction in liver parasitic load was obtained in two different investigations [19,52] using the Balb/c model. In the first study, the vaccine protocol comprised 10 μg of dp 72 in combination with 100 μg of *C. parvum* and 2×10^7 *L. donovani* promastigotes [19]. Similar to the present study, challenge was performed with 7.5×10^6 amastigotes and animals were sacrificed 18 days after infection. An average of 60 LDU was found in livers of saline treated controls [19], while infection levels ranging from 2000 to 3000 LDU were expected [24] for this strain considered to be susceptible [53]. The low degree of infection achieved by controls complicates the interpretation of the dp 72 protective power in this model. In the second investigation, 700 LDU were detected in liver of adjuvant controls and 300 in vaccinated animals [52]. Specificity is however difficult to evaluate since no data about the adjuvant control is presented [54].

Balb/c mice are considered susceptible to experimental visceral leishmaniais, however they do not develop the fatal disease. To our knowledge, the only report of fatal kala-azar in mice described the disease in Swiss albino animals inoculated with 2×10^7 amastigotes [54]. In our study, 6/20 saline treated and 3/20 saponin controls died of visceral leishmaniasis while no death was observed in the GP36 + saponin vaccinated group. This would be the first description of fatal kala-azar in the Balb/c model.

In the present work we showed the strong immunoprotective potential of the *L. donovani* GP36 glycoprotein which might be considered as a potent antigen candidate for a second generation vaccine. As described for recombinant antigens, it might be used at low dosages, and due to the denaturation process included in its isolation protocol it is very stable and can be easily used in large scale field assays.

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EXR. NOTES

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kala-azar in an endemic area of Brazil (Sao Goncalo do Amaranto, RN).
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Protection against canine kala-azar was investigated in naturally exposed dogs of an endemic area in Sao Goncalo do Amaranto, Rio Grande do Norte, Brazil, vaccinated with the fucose mannose ligand (FML)-vaccine of %%%Leishmania%%% donovani . The dog vaccination trial stated in December 1996. A total of 97% of vaccinees were seropositive to FML and 100% showed intradermal reaction to L. donovani %%%lysate%%% , 7 months after vaccination. The absorbency values and size of intradermal reaction were both significantly higher in vaccinees than in controls (ANOVA, P <0.0001). After 2 years, 92% (chi SUP 2 =6.996; P < 0.0025) protection was achieved: only 8% of vaccinees showed mild signs of kala-azar with no deaths while 33% of controls developed clinical or fatal disease. The number of human cases in the area decreased from 15 cases in 1996 to 6 cases in July 1997 and to zero in May 1998. The FML-vaccine induced a significant, long-lasting and strong protective effect against canine kala-azar in the field.

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Human T-cell activation by 14- and 18-kilodalton nuclear proteins of Leishmania infantum

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ABSTRACT: Leishmanial antigens which stimulate T lymphocytes from primed individuals may be candidates for a vaccine. We recently found a significant concordance between the humoral response specific for two proteins from Leishmania infantum promastigotes, p14 and p18, and a positive leishmanin delayed-type hypersensitivity reaction, testifying to the occurrence of cell-mediated immunity. In t

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A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amaranto, RN)

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Abstract

Protection against canine kala-azar was investigated in naturally exposed dogs of an endemic area, vaccinated with the fucose mannose ligand (FML)-vaccine of *Leishmania donovani*. A total of 97% of vaccinees were seropositive to FML and 100% showed intradermal reaction to *L. donovani* lysate, 7 months after vaccination. The absorbency values and size of intradermal reaction were both significantly higher in vaccinees than in controls (ANOVA, $P < 0.0001$). After 2 years, 92% ($\chi^2 = 6.996$; $P < 0.0025$) protection was achieved: only 8% of vaccinees showed mild signs of kala-azar with no deaths while 33% of controls developed clinical or fatal disease. The FML-vaccine induced a significant, long-lasting and strong protective effect against canine kala-azar in the field. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Canine visceral leishmaniasis; Zoonoses; Kala-azar; *Leishmania donovani*

1. Introduction

Zoonotic visceral leishmaniasis (ZVL) is one of the most important emerging diseases. Its etiological agent, (*Leishmania chagasi* or *Leishmania infantum*) infects canids. Peridomestic sandflies acquire the parasite by feeding on the skin of infected foxes and transfer it to wild or domestic dogs. The subsequent transmission to humans, also through sandfly bites, causes human visceral leishmaniasis or kala-azar: a severe disease, lethal if not treated soon after the onset of symptoms. Clinical signs in humans include: malaise, anemia, hepatosplenomegaly, hypergammaglobulinemia, fever, cachexia and progressive suppression of the cellular immune response [1]. About 500 000 human cases of kala-azar are registered annually. A total of 90% of them are in Bangladesh, Brazil, India and Sudan [2]. In Brazil, the highest incidence of disease is found in North-East of the country.

Being a zoonotic disease, the control of canine kala-azar should have an impact by reducing the parasite available to sandflies and therefore reducing the human incidence of disease [1]. The current strategy for control of ZVL, as recommended by the World Health Organization, is based on detection and destruction or treatment of infected dogs, treatment of human cases and vector control [1]. Since the efficacy of this control has shown to be inconsistent, the development of vaccines has been given priority and is considered to be urgent by the World Health Organization.

Immunization against leishmaniasis was achieved in the past by inoculating humans with living parasites that induced localized self-healing cutaneous lesions

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(leishmanization). Since then, a first generation of vaccines against leishmaniasis was composed of formulations including killed parasites (reviewed in Ref. [3]). These formulations were developed against cutaneous but not visceral leishmaniasis and were used in large clinical trials on human populations of endemic areas. Most second-generation vaccines can be divided into three categories according to their composition: live vaccines, defined subunits and crude fractions. Among the recombinant and native antigens tested in murine models, the LACK protein [4], the LPGAP peptides [5], the LeIF protein [6] and the DP72 glycoprotein of *L. donovani* [7] were protective immunogens for mice. Finally, protection results obtained with the third-generation vaccines composed of cDNA encoding leishmanial antigens cloned into an eukaryotic expression vector are still preliminary (reviewed in Ref. [3]). Although a great number of antigens have been tested for protection against the cutaneous disease with in-vitro cell or mouse models, no vaccine against human or canine visceral leishmaniasis is yet available.

In previous reports, the protective potential of a glycoproteic complex purified from promastigotes of *L. donovani* was analyzed [8–12]. The protocol for isolation of this antigen involves aqueous extraction followed by denaturing conditions that determine the further stability of the vaccine [8]. This glycoprotein-enriched fraction was named FML (Fucose mannose ligand) [8]. It is composed of 29% neutral sugar, 44% protein 11% carbohydrate and hexosamines in trace amounts. Among its neutral sugars: fucose (10%), mannose (47%), glucose (30%) and galactose (12%) were identified [8]. The SDS-PAGE analysis of FML disclosed major proteic components with molecular weight ranging from 9 to 95 kDa. Bands of 36 and 55 kDa also showed a positive staining reaction for carbohydrates. The FML aminoacid analysis disclosed the presence of aspartic acid (10%), glutamic acid (16%), alanine (12%), and glycine (10%) as major components [8]. No cross reactivity was observed between FML and the gp63 antigen of *Leishmania* by monoclonal antibodies in ELISA and Western blots [13]. The Western Blot analysis of the FML-reactivity disclosed the GP36 glycoprotein band as the only component reacting with sera of kala-azar patients' sera [14]. No labeling was detected with sera of patients with tegumentar leishmaniasis, Chagas' Disease, or normal sera. The GP36 antigen of FML was shown to be a marker of human kala-azar [14]. A total of 23 hybridomas secreting IgG were obtained from FML immunized Balb/c mice [13]. Twenty-two IgG clones recognized the 36 kDa band of FML. Periodate oxidation of the FML, blotted onto nitrocellulose, prior to reaction with one of monoclonal antibodies raised against the 36 kDa band, abolished the reaction completely [13]. The FML antigen is a potent immunogen to rabbits and mice, present on the parasite surface throughout the life cycle [13] that strongly inhibits the in-vitro infection of murine macrophages by promastigotes and amastigotes of *L. donovani* [8,13] in a species-specific manner [15].

With the FML-vaccine of *Leishmania donovani*, an average protection of 87.7% ($P < 0.01$) and 84% ($P < 0.001$) against kala-azar was achieved in the isogenic CB hamster and Balb/c mouse models, respectively [10,9]. Control animals showed significantly lower levels of anti-FML antibodies, in vitro proliferative splenocyte response and liver LDU ($P < 0.01$ and $P < 0.001$, respectively) with no protective effect. Protection criteria included maintenance of the delayed type of hypersensitivity, the reduction of splenomegaly [10] and parasite load, increase of the in vitro splenocyte proliferation against leishmanial antigen, and increase in anti-FML specific antibody response [9,10]. Also, outbred mice (Swiss Albino) immunized with the FML-vaccine developed higher antibody titers and a significant and specific reduction of parasite load in relation to controls (85%, $P < 0.025$) [12].

In canine kala-azar a strong antibody response is frequent. Therefore, in the field, an anti-FML specific antibody response could indicate the natural infection by *L. chagasi* or the protective response induced by the FML-vaccine formulation. Intradermal reaction (IDR), on the other hand, is known to appear soon after infection in humans and dogs, and turn negative in the advanced kala-azar, indicating the immunosuppressive status of the individual. Therefore, one should expect a transient IDR in naturally infected dogs of saline control and a long-lasting positive IDR in animals that achieved protection to kala-azar after FML-vaccine treatment [16]. In the present study we evaluate the efficacy of the FML-vaccine in the field, in a randomized controlled trial. We used a medium scale population of an endemic area, divided it into vaccine and placebo treated control groups, under conditions that equalized their chance of exposure to natural infection. We used the FML-ELISA assay and the IDR as tools for evaluation of the humoral and cellular dog immune responses.

São Gonçalo do Amaranto is an endemic area for both human and canine visceral leishmaniasis. The overall prevalence of anti-*Leishmania* antibodies disclosed by the FML-ELISA assay in canine sera of this area was 23% (79/343) [17]. The seroreactivity disclosed by an *L. chagasi* immunofluorescent assay (IF) was much lower: 2.9% (10/343), and closely related to the percent of kala-azar symptomatic dogs 2.6% (9/343). All 21 asymptomatic, FML-seropositive animals died from kala-azar in a period ranging from 0 to 6 months after diagnosis, disclosing for the FML-ELISA assay a highly predictive value for the development of both canine and human kala-azar [17,18].

For these reasons we vaccinated uninfected seronegative dogs of São Gonçalo do Amaranto with the FML-vaccine and assayed the efficacy of the vaccine during a 2 year follow up.

2. Material and methods

2.1. Animals and study design

The study was conducted in São Gonçalo do Amaranto, Rio Grande do Norte, Brazil, a populated peri-urban area of Natal, of low socieconomic status where human and canine kala-azar are highly endemic [17,18]. Since December 1996, we have been studying the domestic dog population there. More than 400 animals are being followed serologically and clinically in a longitudinal study.

Since no data was available about vaccination against canine kala-azar in the field, neither with FML-vaccine nor with any other anti-*Leishmania* vaccine, sample size calculations were based on human protection against *Leishmania* achieved after vaccination with a first generation vaccine called Leishvacin, in a previous study done in Brazilian Army conscripts during their training in the Amazon jungle, Brazil [19]. In that report, 67.3% of significant reduction on the incidence of cutaneous leishmaniasis was achieved [19]. In the present study we calculated that on a 95% power to detect 85% of vaccine efficacy (VE) [20] with an α error of 5%, using a two-tailed test, the study required 54 animals per group. 148 domestic dogs in good physical condition were included in this study. The initial serological screening excluded 28 seropositive animals. 120 animals were seronegative both by *L. chagasi* immunofluorescency and FML-ELISA assay and considered eligible. Three animals couldn't be vaccinated since they were absent on vaccination days. The 117 dogs were distributed in two groups: 58 received the FML-vaccine while 59 remained as placebo control treated only with saline. Since previous distribution of seropositive dogs by city blocks was highly heterogeneous, indicating the presence of localized phlebotomine foci, we included vaccinated and placebo control individuals in each house, whenever possible, in order to equalize their degree of exposure to the risk of natural infection. Consent was obtained from the dogs' owners who were informed about the risk of the procedures and the requirement for a 2-year follow-up. In this investigation the collection of biological samples from dogs was performed following the animal experimentation guidelines of the US National Institutes of Health, and removal and sacrifice of animals was done in accordance with the institutional guidelines for the humane use of laboratory animals in order to reduce animal suffering to a minimum.

2.2. Vaccine and vaccination

Vaccine doses, prepared as has been previously described for vaccination of mice [9,12], included the lyophilized FML antigen (1.5 mg), reconstituted in 1 ml NaCl 0.9% sterile saline solution, on each vaccination day. The necessary number of vials were transported to the field with cold packs. Vaccines were administered as three subcutaneous doses in the right flank of the animals at 21 day intervals. The placebo control group was treated with 1 ml sterile saline. Twelve months after vaccination a fourth dose of the vaccine was injected. The FML-vaccine is registered as a Patent: *INPI number*: PI1100173-9 (18.3.97). Federal University of Rio de Janeiro, Brazil.

2.3. Follow-up

Trained field workers, together with our team, made follow-up visits to the vaccinated animals at 2, 7, 13 and 24 months after the complete vaccination. The seroreactivity to FML, IDR response to *L. donovani* promastigote lysate, clinical signs or deaths due to kala-azar were recorded. By the end of the assay (24 months), FML-seropositive animals considered symptomatic were removed from the area, sacrificed by total anesthesia (intravenous Thionembutal, Abbot, São Paulo, Brazil), submitted to autopsy and to parasitological evaluation. We considered as clinical signs of kala-azar: loss of weight, cachexia, alopecia, onycogryphosis, apathy, anorexia, increase of popliteal lymphnode size and ulcerative skin lesions. Liver and spleen were weighed after autopsy of symptomatic dogs in order to determine possible hepato-splenomegaly. The presence of parasites was assayed in their spleen, liver, kidneys, lymphnodes and bone marrow (aspiration of sternal bone) by optical microscopy analysis of Giemsa-stained smears [9]. Also the possible presence of *Leishmania* DNA was assayed in peripheral blood by polymerase chain reaction (PCR) analysis [21].

2.4. Laboratory procedures

The first available prevaccination and post-dose 2, 7, 13 and 24 months serum samples from 117 dogs were tested for the presence of anti-*Leishmania donovani* antibodies by the FML-ELISA assay [17,18]. All dog serum samples were collected from the cephalic vein and conserved in glycerol (1:1, v:v) at -20°C. Isolation and chemical characterization of the Fucose Mannose Ligand antigen, obtained from stationary-growth phase promastigotes of *L. donovani Sudan* (*LD 1S/MHOM/SD/00-strain 1S*) were performed as previously described [8]. The FML (2 μg/well) was solubilized in carbonate buffer (pH 9.6), and used to coat flat-bottom 96-well plates (Haemobag, Ribeirão Preto, SP, Brazil).

Antibodies were detected by peroxidase-labeled protein-A (Sigma, St. Louis, MO) in a 1:1000 dilution; the reaction was developed as described elsewhere [17,18]. The absorbency values at 492 hm were compared using a 1:100 dilution of the serum samples. Results are expressed as mean values of triplicates. The cut-off of the method for the analysis of canine sera was determined according to the Youden test [18]. Briefly, sera from healthy dogs were assayed and the mean absorbency values + 1, 2 or 3 SD were determined. Using these values as cut-off, all samples were analyzed and classified into the following groups: a = disease seropositive; b = disease seronegative; c = healthy seronegative; and d = healthy seropositive. The Youden index was then calculated by means of the formula $j = [a/(a+b) + c/(c+d)] - 1$. The Youden test circumvents the use of an arbitrary cut-off by taking into consideration the possible errors that could lead to negative results for diseased individuals, and positive results for healthy individuals. All serological determinations in this report were done by double-blind tests.

For the indirect immunofluorescent assay (IF), drops of dog blood from the cephalic vein were collected on squares of filter paper, dried and stored at −4°C until analysis. Antibodies were eluted from filter paper with PBS. Dilutions of eluates were incubated with *L. chagasi* promastigotes (Biomanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) plated on slides. The immunofluorescent assay was performed as described elsewhere [17]. Reactions were considered positive if fluorescent at a 1:40 dilution.

Blood and bone-marrow samples were collected in EDTA-tubes for DNA isolation and PCR analysis [21]. Genomic DNA was extracted from 500 μl of whole blood using the RapidPrep™ Kit (Pharmacia Biotech-Upsala, Sweden) following the manufacturer's instructions. The DNA eluted from the chromatographic procedure was precipitated with sodium acetate and ethanol, and resuspended in 10 μl of TE (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). The hot start PCR was performed with a pair of oligonucleotides that anneal to the origin of replication of both strands of the mini circle molecules which are one of the components of the genus *Leishmania* mitochondrial DNA (kDNA). Primer A was 5′(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACCAACCCC and primer B was 5′GGGGAGGGGCGTTCTGCGAA [18]. These oligonucleotides amplify the conserved region of the minicircle molecule, using 35 cycles of 94/50/72°C. The reactions were performed using 200 ηg of each nucleotide, 200 μM of a dNTPs mixture, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3 and 2.5 U of Taq polymerase (Perkin-Elmer, Branchburg, NJ, USA). This assay detects kDNA from less than one organism by amplifying a product of a conserved region, and thus is specific for the genus *Leishmania*. The 120 bp amplification products were analyzed by electrophoresis on 2% agarose gels followed by ethidium bromide staining and visualization under UV light. All reactions (every eight samples) were performed with two extra tubes: one negative control where no DNA was added to the mixture and one positive control, using DNA isolated from cultured promastigote parasites of *Leishmania donovani Sudan* (LD 1S/MHOM/SD/00-strain 1S). Irrelevant DNA from other infectious and non-infectious human diseases was included as negative controls [21].

2.5. Delayed type hypersensitivity (intradermal reaction to promastigote lysate)

This was determined by injecting dogs intradermally, in the inner aspect of the right hind leg, with 0.1 ml of *L. donovani* freeze-thawed antigen containing 200 μg protein in NaCl 0.9% sterile saline solution (10^8 stationary phase promastigotes/ml). The left hind leg received only 0.1 ml saline. Measure of the increase of intradermal reaction was performed 24, 48 and 72 h after antigen injection. Indurated areas were marked and each time, the values of the saline control were subtracted from the reaction due to the *Leishmania* antigen. Reactions showing diameters ≥5 mm were considered positive.

2.6. Statistical analysis

Means were compared by a standard t test and ANOVA analysis, simple factorial test (SPSS). Chi-square and Fisher's exact tests were used in comparing proportions [22]. Correlation coefficient analysis was determined by a Pearson bivariate, two-tailed test of significance (SPSS).

3. Results

3.1. Antibody and IDR response in vaccinated and control dogs

A pre-immunization census performed in São Gonçalo do Amaranto, RN, excluded 8/148 dogs that showed a positive reaction in the *L. chagasi* IF and 28/148 dogs seropositive in FML-ELISA indicating a 5.4 and 18.9% seroprevalence, respectively in both assays. Among the last 28 were the 8-IF-positive specimens already mentioned.

Thus, prior to vaccination, all dogs included in the experiment were seronegative by both IF and FML-ELISA assays. Between January and February 1997, 117 dogs received their three vaccine or placebo injections. Antibody rises against FML antigen were detected in FML-vaccinated animals, 2 months after

vaccination (Table 1). The increase was significantly higher than that detected in saline controls which was probably due to exposure to natural infection by *L. chagasi*. 7 months after vaccination 97% of vaccinated animals already showed seropositivity, maintaining this saturating value throughout the first year of the experiment with no apparent decay. A booster was given at the thirteenth month (after sera collection) and 100% of the animals were seropositive 24 months after complete vaccination. The differences with saline control were highly significant along the whole period (Table 1), even though the proportion of seropositive animals also increased in the controls, indicating natural infection by *L. chagasi*.

Likewise, the proportion of positive IDR responses to the f/t lysate of *L. donovani* was also significantly increased in vaccinated animals over saline controls, starting from 2 months after complete vaccination (Table 1). A total of 100% of animals were IDR positive by the end of the first year of the experiment. Control animals treated with saline developed lower proportions of IDR reactions, during the first 7 months of the experiment, probably related to the presence of active leishmanial natural infection. In contrast to what was detected in serology, the IDR positivity showed a slight decrease, 13 months after vaccination, indicating the expected initiation of suppressive cellular immune response in the exposed animals.

The anti-FML humoral response induced by the FML-vaccine could be analyzed not only by means of proportion of seroconversion but also by the increase in absorbency values. Fig. 1 shows the evolution of the anti-FML total antibody levels achieved in the vaccinated and control animals. The results represent the mean $\pm$ SE values of absorbency readings at 492 hm, for total anti-FML immunoglobulins, in 1:100 diluted serum of each seropositive animal. The humoral response was significantly higher in the FML-vaccine group than in controls treated with only saline at all tested times (ANOVA analysis, $P < 0.0001$ differences for treatment, F: 244,782 and $P < 0.0001$ in differences for time, F: 35,386). A plateau in absorbency values was achieved soon after the booster dose (13 months after vaccination) with no decline detected until the end of the second year of the experiment.

Also, the increase in the average size of the IDR to promastigote lysate was evaluated (Fig. 2) in the vaccinated and control animals. The results represent the mean $\pm$ SE values of skin reaction diameters (mm) of all reactive animals, 24 h after antigen injection. The IDR response was significantly higher in the FML-vaccine group than in saline treated controls at all tested times (ANOVA analysis, $P < 0.0001$ differences for treatment, F: 234,109 and $P < 0.0001$ in differences for time, F: 12,708).

The mean value $\pm$ SE of serum absorbencies at the different time points (2, 7, 13 and 24 months after vaccination) was 0.432 ± 0.125 (Fig. 1). The corresponding mean value $\pm$ SE of skin reaction sizes was 5.083 ± 0.167 mm (Fig. 2). These results demonstrate the exposure of animals in this endemic area to a constant natural infection pressure. The homogeneity of positive response in serology to FML and IDR to promastigote lysate demonstrate the strong potential of FML vaccine that induced both, humoral and cellular immune responses in a highly heterogeneous vaccinated dog population composed of outbred animals of different ages, sexes and races. Correlation coefficient analysis between the anti-FML humoral and IDR response was determined on a Pearson bivariate, two-tailed test of significance. The two variables were shown to be highly correlated ($n = 657$; $P < 0.001$).

3.2. Prevention of kala-azar and Leishmania infection

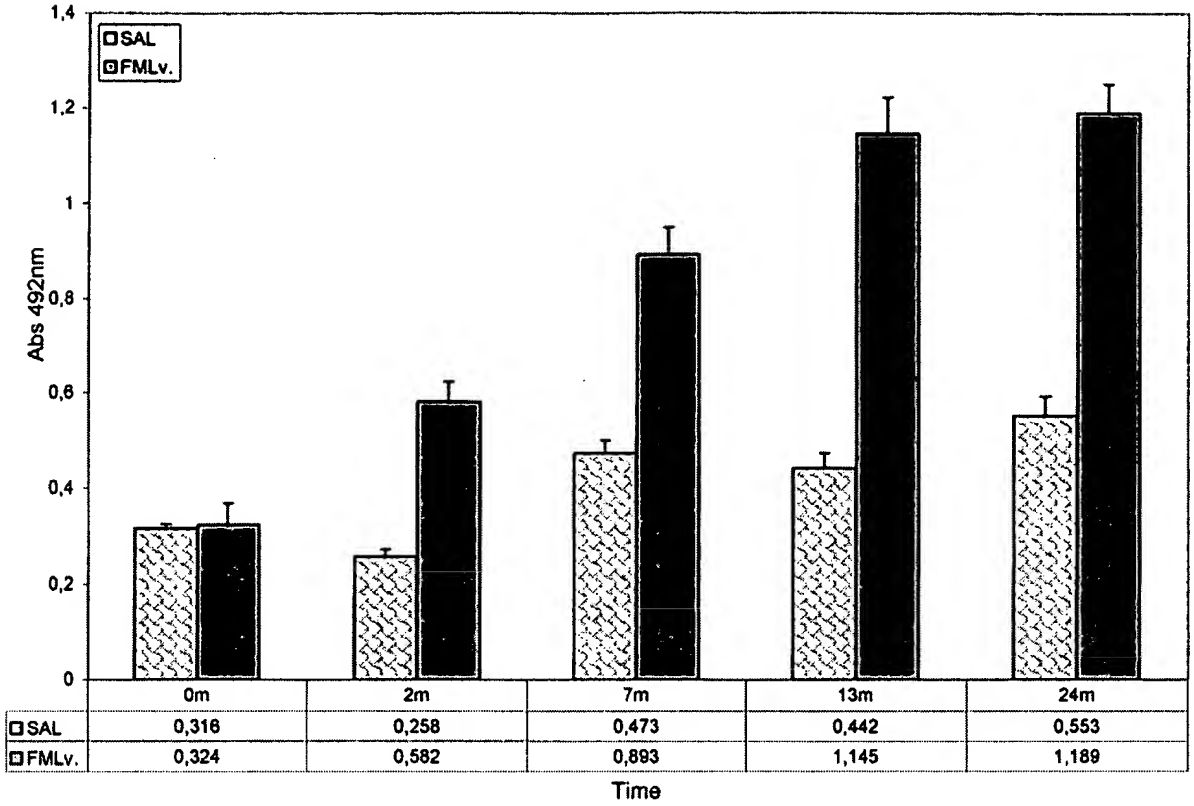
Four placebo-treated control dogs died of serologically, clinically and parasitologically confirmed kala-azar in the control group, by the end of the first year of the experiment, while no death was recorded in vaccinated animals during the first or second year period.

By the end of the assay (24 months) FML-seropositive animals considered symptomatic were removed from the area, analyzed for anti-FML antibodies and IDR response, sacrificed, submitted to autopsy and to parasitological evaluation (Table 2). The presence of parasites was assayed microscopically in Giemsa-

Table 1
Seropositivity in the FML-ELISA assay of vaccinated and control dogs[a]

Protective response	2 months			7 months			13 months			24 months		
	Sal	FMLv	*P<	Sal	FMLv	P<	Sal	FMLv	P<	Sal	FMLv	P<
FML-ELISA+	14	62	0.005	45	97	0.005	40	97	0.005	68	100	0.0025
IDR+(24 h)	15	58	0.005	37.5	97	0.005	25	100	0.005	32	94	0.005
IDR+(48 h)	6	12	0.05	15	82.3	0.005	7.7	91	0.005	14	94	0.005

[a] NOTE. Values represent percent of positive reactions in samples collected 2, 7, 13 and 24 months after vaccination. Sal: percent of saline-treated controls; FMLv: percent of FML-vaccine treated animals; IDR: intradermal reaction to *L.* (*L.*) *donovani* promastigote lysate (200 μg of protein). Significance of proportions compared by Chi square and Fisher's exact tests.



	0m	2m	7m	13m	24m
SAL	0,316	0,258	0,473	0,442	0,553
FMLv.	0,324	0,582	0,893	1,145	1,189

Fig. 1. Evolution of the anti-FML antibody absorbency values with time in naturally exposed vaccinated dogs and saline treated controls. The results represent the mean ± SE values of absorbency readings at 492 hm, for total anti-FML immunoglobulins, in 1:100 diluted serum of each seropositive animal. Sal: saline-treated controls; FMLv: FML-vaccine treated animals.

stained smears and in peripheral blood by PCR analysis. In the saline controls, five/six animals were FML-seropositive already. While the weakly positive IDR in two animals indicated recent infection by *Leishmania*, the skin reaction was absent, suppressed in four/six dogs. Although amastigotes were absent from all smears, the presence of infection was confirmed by the detection of leishmanial DNA in peripheral blood of all the six oligosymptomatic animals (Table 2).

Conversely, all FML-vaccinated individuals were positivite by serology and IDR, indicating the long-lasting protective status against the disease. Indeed, amastigotes were absent from all smears and infection by *Leishmania* was only confirmed in three/five symptomatic subjects. The anorexia and alopecia detected in the 2 PCR negative dogs was, therefore, not related to kala-azar infection (Table 2).

Fig. 3 shows the proportion of protection against kala-azar achieved in the FML-vaccinated dogs. The rate of infection was significantly higher in saline controls. Indeed, 33% of the animals developed either clinical or fatal disease while only 8% of vaccinated dogs showed mild signs of kala-azar with no deaths. This means that 92% protection against kala-azar was achieved after FML vaccination. The difference between the groups was highly significant ($\chi^2 = 6.446$; $P < 0.0025$).

Taken together, the effect on humoral and cellular immune responses, survival after kala-azar infection and decline in parasitological and clinical signs of disease, the FML-vaccine induced a significant, long-lasting and strong protective effect against canine visceral leishmaniasis in the field.

4. Discussion

Vaccine efficacy is measured by calculating the incidence rates of disease among vaccinated and unvaccinated subjects and determining the percentage reduction in the incidence rate of disease between the two populations [20]. In practice, vaccines are neither perfectly effective nor totally ineffective. Human measles vaccine and veterinary rabies vaccines, for instance, are 80–95% [20] and 80–90% effective, respectively, when appropriately administered.

The ideal vaccine efficacy study is a clinical trial starting with subjects susceptible to the disease. In a double blind placebo control trial (Phase III), half of the subjects receive vaccine and half receive placebo. Both groups are prospectively followed [20]. Regarding the FML-vaccine, the trials performed in CB hamsters,

Balb/c and Swiss albino mice correspond to Phase I-IIa trials [9–12]. It was possible to gather information about safety, immunogenicity and efficacy of the vaccine, both before and after artificial challenge, using a small scale population (10–100). An 84–87.7% average of significant and specific protection ($0.01 < P < 0.001$) was achieved in these models [9,10,12]. Furthermore, the standardization trial performed on vaccinated and experimentally infected dogs, also represents a Phase IIa trial in a more appropriate experimental model [16].

In the present work, a Phase III trial of the FML-vaccine was developed, on a large scale dog population of an endemic area composed of vaccinees and placebo treated animals that were prospectively studied for a 2 year period. A total of 92% significant and long-lasting protection against canine kala-azar was obtained with this formulation. This means that the next step in FML-vaccine development could be the development of either a new Phase III or Phase IV trials. Protection against canine kala-azar due to FML-vaccine is also probably related to the reduction of human disease in this area. Indeed, São Gonçalo district represented until 1996 the origin of 6% of total human kala-azar cases in Rio Grande do Norte. This dog vaccination trial started at December 1996. The number of human cases in this area decreased from 15 cases in 1996 to 6 cases up to July 1997 and to zero until May 1998.

The control of visceral leishmaniasis in North-East Brazil in the past 30 years has centered on the detection of infected dogs by serological enquires and elimination of seropositive dogs [23]. Usually 50% of seropositive dogs had amastigote-like organisms [23]. Some problems, however, still need to be solved in order to improve the control program efficacy. The commonly used method for control programs is immunofluorescence (IF). It usually shows high correlation with parasitologic confirmation of infection. Due to its low sensitivity, this method underestimates the true prevalence of canine infection, missing some cases. It is known that, in endemic areas, some dogs are seropositive and infected but asymptomatic or oligosymptomatic [24–26], and others, also infected, spontaneously recover [27]. It is important to note that in the endemic area, the FML-vaccine not only reduced the number of deaths due to kala-azar but the number of poly and oligosymptomatic dogs as well. This fact is relevant since naturally infected dogs, both asymptomatic and oligosymptomatic, proved to be infective for sandflies [24].

The seropositivity in IF and FML-ELISA disclosed in the pre-immunization census showed correlation with

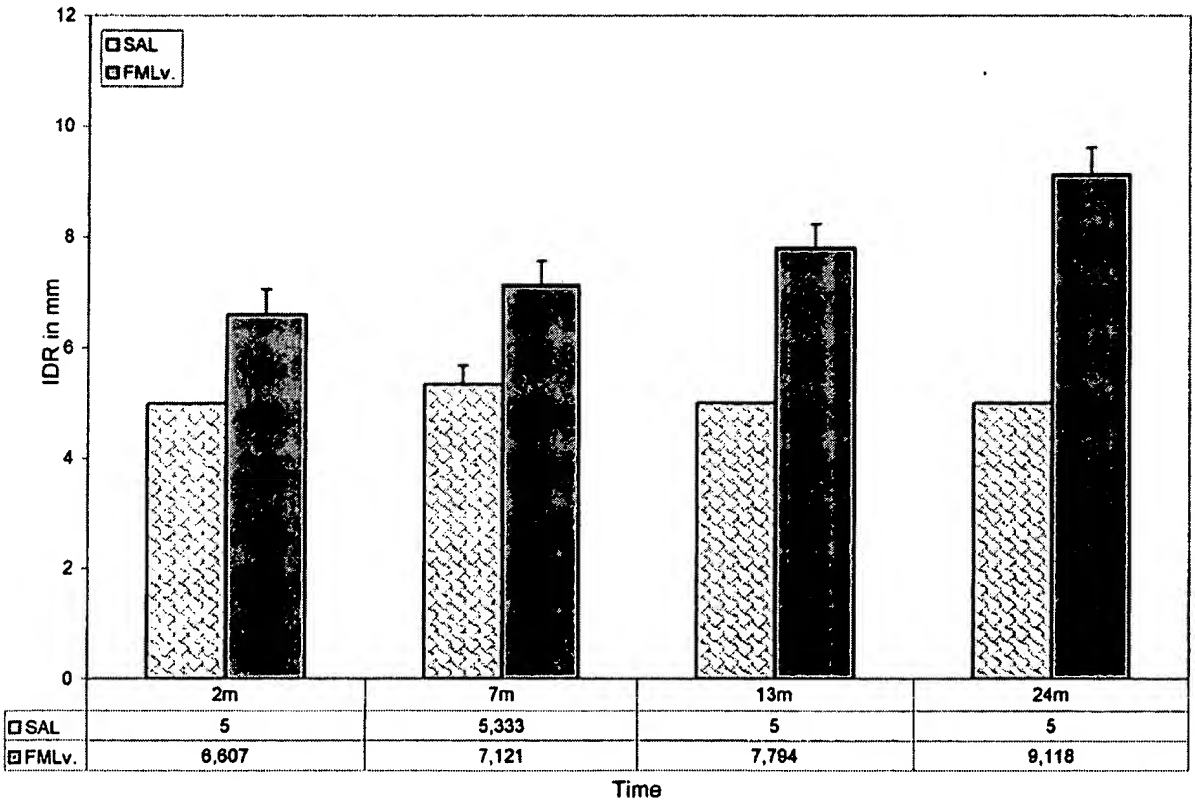


Fig. 2. Evolution of the size of intradermal skin reaction to *L. donovani* lysate with time in naturally exposed vaccinated dogs and saline treated controls. The results represent the mean ± SE values of skin reaction diameters (mm) of all reactive animals, 24 h after antigen injection. Sal: saline-treated controls; FMLv: FML-vaccine treated animals.

Table 2
Parasite confirmation in symptomatic dogs 24 months after vaccination with the FML-vaccine[a]

Group	FML-ELISA* (Abs 492 nm)	IDR** (mm)	Symptoms	PCR	Amastigotes
Saline	0.564	–	Disseminated ulcer	+	–
Saline	0.936	–	Onycogryphosis	+	–
Saline	0.484	–	Isolation	+	–
Saline	0.386	5	Alopecia	+	–
Saline	0.766	–	Onycogryphosis	+	–
Saline	1.088	5	Alopecia	+	–
FML-v	1.618	10	Onycogryphosis	+	–
FML-v	1.760	10	Anorexia	–	–
FML-v	1.177	10	Alopecia	+	–
FML-v	0.659	10	Onycogryphosis	+	–
FML-v	1.439	5	Alopecia	–	–

[a] NOTE. * These are the absorbency values of sera in the FML-ELISA assay. Sera of symptomatic dogs were collected before sacrifice. Absorbency values higher than 0.435 were considered positive results. ** These are the diameters of intradermal reactions after 24 h injection of promastigote lysate antigen. Reactions were considered positive if ≥ 5 mm. Saline: percent of saline-treated controls; FMLv: percent of FML-vaccine treated animals; IDR: intradermal reaction to *L. donovani* promastigote lysate (200 μg of protein); PCR: polymerase chain reaction performed on genomic DNA extracted from 500 μl of whole blood and amplified using a pair of oligonucleotides that anneal to the origin of replication of both strands of the mini circle molecules which are one of the components of the genus *Leishmania* mitochondrial DNA (kDNA).

results previously obtained in the same area (2.6 and 23%, respectively) [17] and in Jequié, Bahia, another Brazilian kala-azar endemic region (23.5%) [28]. However, two years after vaccination, seropositivity to FML in saline treated controls was 68%. The proportion of deaths due to confirmed kala-azar in the control group was 6.8%. This value is not different from the usual expected in this area is 2–3% ($\chi^2 = 0.0003$; $P > 0.05$).Therefore, the increase in seropositivity was not related to any change in risk of infection. Regarding human tegumentar leishmaniasis, it was already proved that the amount of antigen utilized in the MST (Montenegro skin test), used for diagnostic purposes, or as a criterion for inclusion into vaccination or immunological assays, is in fact capable of inducing a new or modifying a pre-existing, immune response to *Leishmania*. Indeed, a first skin test in human naive individuals induced positivity in a second one (4/5) and an increase in anti-*Leishmania* antibody titers on ELISA assay (2/6) [29]. The skin test is used for the screening of individual candidates to participate in a vaccination assay against tegumentar leishmaniasis. Only subjects that show negative reactions, meaning no previous contact with *Leishmania*, would be included in the experiment. This means that the relatively small amount of antigen (4 μg of protein) injected for the skin test leads to a specific sensitization. In our dog vaccination experiment, initial screening was performed with a non-invasive tool: the FML-ELISA assay. The skin test was only used for monitoring the protective cellular immune response. As we previously stated, intradermal reaction is a clear marker of protection in human or canine kala-azar. Seropositivity, on the other hand, would characterize both the presence of *Leishmania* infection and a humoral post-vaccination response. In the present investigation, the proportion of positivity and the size of the skin reaction was significantly higher in vaccinees than in placebo controls throughout the two year period. However, the successive *L. donovani* antigen injections performed for skin testing could be partially responsible for the increase in seropositivity of the placebo group (68%) over the expected prevalence for this endemic area (2–3%). The maintenance of the same incidence of the disease in the placebo group showed that these injections, while inducing an antibody response, did not elicit any effective protective response against kala-azar.

In the present study, the characterization of an in-vitro lymphoproliferative response to leishmanial antigen was not achieved. The endemic area of São Gonçalo do Amaranto is more than 2000 km away from our laboratory and according to the sanitary control policy, the removal of *Leishmania chagasi* infected dogs from endemic to non-endemic areas is forbidden, in order to avoid the possibility of dissemination of disease. However, in our experimental kennel, we were able to analyze the lymphoproliferative response of mongrel dogs immunized with three doses of the FML-vaccine, and experimentally challenged with 10^8 amastigotes of *L. donovani* through the i.v. route. The protective response was monitored monthly, up to 540 days after infection. The proliferative in vitro response of blood mononuclear cells to FML, GP36 and promastigote lysate antigens was evaluated. The differences between the in vitro proliferative response of vaccinees and controls were not significant. Both groups developed a proliferation peak between 180 and 300 days after infection. The IDR response was positive and significantly higher in vaccinees than in saline

controls ($P < 0.01$) while clinical signs of disease were more evident in saline controls ($P < 0.001$) [16]. In agreement with our results, Binhazim et al. (1993), demonstrated a similar proliferative response in normal controls and infected dogs [30]. Abranches et al. (1991) did not show differences in lymphoblastic proliferation between infected and uninfected dogs, when treated with ConcanavalinA e Phytohaemagglutinin mitogens. However, the proliferative response against total antigens of *L. infantum* e *L. donovani* was supressed in six/seven infected individuals [31]. Pinelli et al. (1994) showed that asymptomatic or resistant dogs responded to *L. infantum* antigen both by lymphocyte proliferation assays and by the IDR reaction [32]. In contrast, symptomatic dogs failed to respond to parasite antigen both in-vitro and in-vivo. Furthermore, higher levels of IL-2 and tumor necrosis factor were found in supernatants from stimulated mononuclear cells from asymptomatic dogs compared with those of symptomatic and control dogs [32]. The characterization of the cytokine response of cells derived from dogs treated with FML-vaccine is under progress.

Costa et al. (1996) during a field trial in Montes Claros, Brazil, described a higher incidence of *L. chagasi* infections in Dobermans, followed by Fox Terrier and Pinscher dogs [33]. In the metropolitan region of Lisbon, Portugal, Abranches et al. (1991) pointed out the Doberman, followed by German Shepherd, as the most affected breeds [34]. Therefore, different suceptibilities to disease are expected in relation to the genetic background. The dogs included in our assay, however, correspond mostly to the mongrel kind (97.5%), being then a genetically highly heterogeneous population. Indeed, only one Doberman and two German Shepherd dogs were included among the 120 animals considered eligible for the assay, making up 2.5% of the whole population. Therefore, the analysis of the impact of the FML-vaccine protection in relation to any genetic basis of susceptibility to visceral leishmaniasis, is not possible. All animals included in this study were healthy and well nourished, since they were domestic pets. Also, they were at least 4 months old, by the beginning of the experiment, in order to guarantee that any potential seroreactivity to leishmanial antigens would not be related to maternal antibodies acquired during breast feeding. The initial number of dogs submitted to serological screening for anti-*Leishmania* antibodies included a majority of males, (95/148) representing 64% of the whole population. One of the possible reasons for this significant difference ($P < 0.001$), found in the endemic area, could be a sex related susceptibility to the disease, e.g. male dogs being more resistant to kala-azar. Two pieces of evidence supported this hypothesis: (1) the first serological screening performed in the area disclosed higher proportions of seropositivities to FML among females, indicating previous infection, and (2) by the end of the assay, higher proportions of kala-azar confirmed cases were found in females. However, the statistical analysis of these differences showed no significance (seropositivity: 12/54 females; 17/95 males; kala-azar cases: 6/37 females; 7/67 males). Future studies with larger samples could clarify this point.

To our knowledge, there is only one reference in literature about canine vaccination against visceral leishmaniasis. Dunan et al. (1989) used a *L. infantum* vaccine composed of semi-purified lyophilized protein preparation of a mol. wt. ranging from 94 to 67 kDa [35]. The authors achieved, during the first year of experiment, a rate of infection significantly higher ($P < 0.05$) in vaccines (17) than in control dogs (5), but this difference disappeared during the second year ($P = 0.44$). These results led the authors to the conclusion that this vaccine, while effective in murine models, developed no protection against canine kala-azar in the field. Hence, the present investigation constitutes the first report of a canine vaccine for visceral leishmaniasis that elicits a long-lasting and significant protective effect.

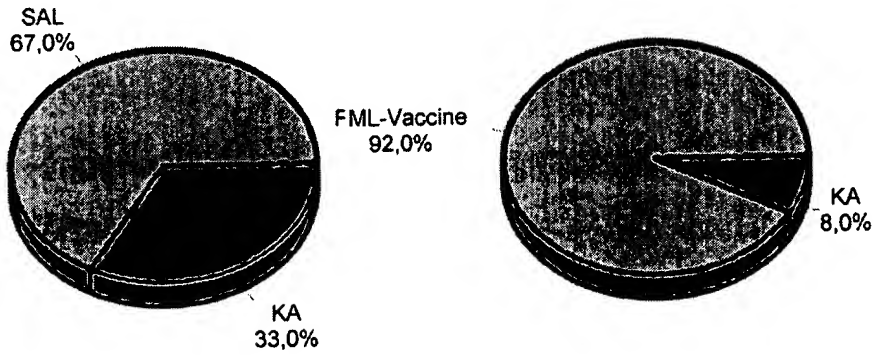


Fig. 3. Proportion of protection against kala-azar achieved in the FML-vaccinated dogs. Sal: saline-treated controls; FMLv: FML-vaccine treated animals.

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EXR. NOTES

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organisms, a study was undertaken to examine the role of IL-12 in the immune regulation of human visceral %%%leishmaniasis%%% (VL). Human (Hu) VL is associated with immune dysfunction and the appearance of IL-12 mRNA, not present in healed individuals. We found that PBMC from treated VL patients produced both IL-12 p40 and IFN-gamma in response to in vitro stimulation with %%%Leishmania%%% donovani. The production of both IL-12 p40 and IFN-gamma were interdependent and were abrogated by the addition of exogenous Hu rIL-10. In contrast, PBMC from active VL patients did not produce IL-12 p40 or IFN-gamma in response to L. donovani %%%lysate%%%. Neutralizing anti-IL-10 mAb led to the enhancement of IFN-gamma production by active VL PBMC cultured with L. clonovani %%%lysate%%%, and this enhanced IFN-gamma production was blocked by anti-IL-12 mAb. The addition of exogenous Hu rIL-12 to PBMC from active VL patients resulted in the augmentation of IFN-gamma in response to L. donovani %%%lysate%%%. Therefore, treatment of active VL patient PBMC with anti-IL-10 or IL-12 shifted the response toward a Th1-type response with the production of IFN-gamma. These results indicate that IL-12 may play an important role in the regulation of the cellular immune responses in Hu VL.

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A recombinant %%%Leishmania%%% antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukin 12
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ABSTRACT: %%%Leishmania%%% braziliensis causes cutaneous and mucosal %%%leishmaniasis%%% in humans. Most patients with cutaneous %%%leishmaniasis%%% heal spontaneously and may therefore have developed protective immunity. There appears to be a mixed cytokine profile associated with active cutaneous or mucosal disease, and a dominant T helper (Th)1-type response associated with healing. %%%Leishmanial%%% antigens that elicit these potent proliferative and cytokine responses from peripheral blood mononuclear cells (PBMC) are now being identified. Herein, we report on the cloning and expression of a L. braziliensis gene homologous to the eukaryotic ribosomal protein eIF4A (LeIF) and patient PBMC responses to rLeIF. Patients with mucosal and self-healing cutaneous disease had significantly higher proliferative responses than those with cutaneous lesions. Whereas the parasite %%%lysate%%% stimulated patient PBMC to produce a mixed Th1/Th2-type cytokine profile, LeIF stimulated the production of interferon gamma (IFN-gamma), interleukin 2 (IL-2), and tumor necrosis factor alpha but not IL-4 or IL-10. Recombinant LeIF (rLeIF) downregulated both IL-10 mRNA in the "resting" PBMC of %%%leishmaniasis%%% patients and LPS-induced IL-10 production by patient PBMC. rLeIF also stimulated the production of IL-12 in cultured PBMC from both patients and uninfected individuals. The production of IFN-gamma by patient PBMC stimulated with either rLeIF or parasite %%%lysate%%% was IL-12 dependent, whereas anti-IFN-gamma monoclonal antibody only partially blocked the LeIF-induced production of IL-12. In vitro production of both IFN-gamma and IL-12 was abrogated by exogenous human recombinant IL-10. Therefore, we have identified a recombinant

A Recombinant *Leishmania* Antigen that Stimulates Human Peripheral Blood Mononuclear Cells to Express a Th1-Type Cytokine Profile and to Produce Interleukin 12

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Summary

Leishmania braziliensis causes cutaneous and mucosal leishmaniasis in humans. Most patients with cutaneous leishmaniasis heal spontaneously and may therefore have developed protective immunity. There appears to be a mixed cytokine profile associated with active cutaneous or mucosal disease, and a dominant T helper (Th)1-type response associated with healing. Leishmanial antigens that elicit these potent proliferative and cytokine responses from peripheral blood mononuclear cells (PBMC) are now being identified. Herein, we report on the cloning and expression of a *L. braziliensis* gene homologous to the eukaryotic ribosomal protein eIF4A (LeIF) and patient PBMC responses to rLeIF. Patients with mucosal and self-healing cutaneous disease had significantly higher proliferative responses than those with cutaneous lesions. Whereas the parasite lysate stimulated patient PBMC to produce a mixed Th1/Th2-type cytokine profile, LeIF stimulated the production of interferon γ (IFN-γ), interleukin 2 (IL-2), and tumor necrosis factor α but not IL-4 or IL-10. Recombinant LeIF (rLeIF) downregulated both IL-10 mRNA in the "resting" PBMC of leishmaniasis patients and LPS-induced IL-10 production by patient PBMC. rLeIF also stimulated the production of IL-12 in cultured PBMC from both patients and uninfected individuals. The production of IFN-γ by patient PBMC stimulated with either rLeIF or parasite lysate was IL-12 dependent, whereas anti-IFN-γ monoclonal antibody only partially blocked the LeIF-induced production of IL-12. In vitro production of both IFN-γ and IL-12 was abrogated by exogenous human recombinant IL-10. Therefore, we have identified a recombinant leishmanial antigen that elicits IL-12 production and Th1-type responses in patients as well as IL-12 production in normal human PBMC.

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Leishmania are obligate intracellular protozoan parasites of macrophages that cause a spectrum of human diseases, including self-healing skin lesions, diffuse cutaneous and mucosal manifestations, or severe visceral disease (1). *Leishmania braziliensis* commonly causes localized cutaneous leishmaniasis (CL)[1]. Most patients with CL heal spontaneously (2). However, a chronic mucosal leishmaniasis (ML) develops in some infected individuals, with severe and progressive destruction of the nasal, oral, and/or pharyngeal mucous membranes (3, 4). Patients with CL generally have positive delayed hypersensitivity and in vitro proliferative responses to leishmanial Ag during both active and cured infection. These responses are often exaggerated in ML patients (5, 6).

The induction of Th1 effector cells capable of activating macrophages to a microbicidal state is necessary for eliminating these intramacrophage parasites. In experimental leishmaniasis, immunological interventions to direct $CD4^+$ T cell response must be administered before the establishment of Th1 or Th2 effector subsets. IL-12, which facilitates the development of Th1 responses by stimulating the production of IFN-γ while downregulating the production of IL-4, has been demonstrated to cure susceptible BALB/c mice against *L. major* infection (7, 8), whereas neutralization of IL-12 made resistant mice susceptible to infection (8). Although neutralization of IFN-γ abrogates the natural resistance of C3H/HeN mice to infection with *L. major* (9), by itself, IFN-γ did not confer protection to susceptible mice (10).

[1] *Abbreviations used in this paper:* CL, cutaneous leishmaniasis; LeIF, *L. braziliensis* homologue of the eukaryotic initiation factor 4A; ML, mucosal leishmaniasis; rAg, recombinant antigen; rLeIF, recombinant LeIF.

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The determination of cytokine profiles in PBMC from leishmaniasis patients with different clinical presentations as well as the identification of defined Ag that induce and elicit cell-mediated immune responses is important to our understanding of the nature of immune responsiveness during infection, as well as the potential identification of vaccine molecules. The elucidation of cytokines associated with Th1 and/or Th2 responses by defined *L. braziliensis* Ag in patients with leishmanial infections has not been previously described. Because the course of human infection with *L. braziliensis* is variable, ranging from self-healing infection to chronic disease, it is an excellent system in which to study immunoregulatory aspects of leishmaniasis, including the effects of parasite Ag on host responses. In the present study, *Leishmania*-specific PBMC responses in patients representing a spectrum of *L. braziliensis* infection were analyzed. A leishmanial Ag, *L. braziliensis* homologue of the eukaryotic initiation factor 4A (LeIF), capable of stimulating strong Th1-type responses in leishmaniasis patients' PBMC as well as IL-12 production in patient and nonpatient cells, was identified.

Materials and Methods

Parasites. *L. braziliensis* (MHOM/BR/75/M2903), *L. guyanensis* (MHOM/BR/75/M4147), *L. amazonensis* (IFLA/BR/67/PH8), *L. chagasi* (MHOM/BR/82/BA-2, C1 and MHOM/BR/84/Jonas), *L. donovani* (MHOM/Et/67/HU3), *L. infantum* (IPT-1), *L. major* (LTM p-2), *L. tropica* (1063C), *Trypanosoma cruzi* (MHOM/CH/00/Tulahuen C2), and *T. brucei* (TREU 667) were used (11). Promastigotes of *Leishmania* and epimastigotes of *T. cruzi* were cultured in axenic media. *L. chagasi* and *L. amazonensis* amastigotes were obtained from spleens of Syrian hamster and footpads of BALB/c ByJ mice, respectively.

Library Construction and Isolation of Genomic Clones. A genomic expression library with sheared DNA from *L. braziliensis* (MHOM/BR/75/M2903) was constructed in bacteriophage ZAPII (Stratagene, La Jolla, CA). The expression library was screened with *Escherichia coli* adsorbed sera from patient with ML due to infection with *L. braziliensis*. A plaque containing an immunoreactive recombinant antigen (rAg) (LeIF.1) was purified, the pBSK(−) phagemid excised, nested deletions generated using Exonuclease III for single-stranded template preparations and sequencing (12). Clone pLeIF.1 lacked the first 48 amino acid residues (144 nucleotides) of the full-length sequence. The insert was subsequently used to isolate the full-length genomic sequence.

Expression and Purification of Recombinant LeIF (rLeIF). The 45-kD rAg of the LeIF.1 genomic clone was purified from 500 ml of isopropyl β D-thiogalactoside (IPTG)-induced cultures (12). Inclusion bodies were isolated and sequentially washed in TNE (50 mM Tris, pH 8.0, 100 mM NaCl, and 10 mM EDTA) containing 2, 4, and 8 M urea. Fractions containing solubilized rAg (usually the 8 M cut) were pooled, dialyzed against Tris-buffered saline (TBS), and concentrated by precipitation with 30% ammonium sulfate. Purification to homogeneity was accomplished by preparative SDS-PAGE electrophoresis, followed by excision and electroelution of the rAg as described. All Ag used in our studies had negligible (<10 pg/ml) endotoxin in a Limulus amebocyte assay.

Production of Rabbit Antiserum against rLeIF. An adult rabbit (New Zealand White; R & R Rabbitry, Stanwood, WA) was immunized with 200 μg s.c. of purified rLeIF in IFA (GIBCO BRL, Gaithersburg, MD) with 100 μg of muramyl dipeptide (adjuvant peptide; Calbiochem-Novabiochem Corp., La Jolla, CA), followed by a s.c. boost 4 wk later with 100 μg of the rAg in IFA alone. After 3 wk, the rabbit was boosted with 25 μg i.v. of rLeIF in saline, and serum collected 1 wk later.

Cell Lysate. Parasite and mammalian cell lysates were prepared by freeze–thaw lysis of pellets in SDS sample buffer without glycerol or β-ME. Insoluble material was separated from the supernatant by centrifugation at 10,000 rpm. Protein concentrations were determined using a bicinchoninic acid protein assay kit (BCA; Pierce Chemical Co., Rockford, IL).

Immunoblot Analysis. 5–10 μg of parasite or cell extracts or 0.5–1.0 μg of rAg were separated on 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Reactivities of the antisera were assessed as previously described (12) using ^{125}I-protein A, followed by autoradiography.

Patients. Peripheral blood was obtained from individuals living in an area endemic for *L. braziliensis* (Corte de Pedra, Bahia, Brazil) in which epidemiological, clinical, and immunological studies of leishmaniasis have been performed for over a decade (3). Diagnosis was made by clinical findings associated with at least one of the following: parasite isolation, leishmanial skin test, or positive serology. An epidemic in the field area without access to glucantime enabled us to detect patients who had healed spontaneously (self-healing).

PBMC Culture, Proliferation, and Cytokine Assays. Peripheral blood from *Leishmania* infected or uninfected individuals were collected and PBMC isolated from whole blood by density centrifugation through Ficoll (Pharmacia Biotech Inc., Piscataway, NJ). For in vitro proliferation assays, 2–4 × 10^5 cells/well were cultured in complete medium (RPMI 1640 supplemented with gentamycin, 2-ME, L-glutamine, and 10% screened pooled A+ human serum; Trimar, Hollywood, CA) in 96-well flat bottom plates with or without 10 μg/ml of the indicated Ag or 5 μg/ml PHA (Sigma Immunochemicals, St. Louis, MO) for 5 d. The cells were then pulsed with 1 μCi of [^{3}H]thymidine for the final 18 h of culture. Data are represented as mean cpm of triplicate cultures and the stimulation index (SI) defined as mean cpm of cultures with Ag/mean cpm of cultures without Ag. For determination of cytokine production, PBMC were cultured at 1–2 × 10^6 cells/ml in medium containing endotoxin-free FCS (Cellect Gold; ICN Biomedicals, Inc., Costa Mesa, CA) with or without leishmanial Ag and in the presence or absence of human (Hu) rIL-10 (10 ng/ml, DNAX, Palo Alto, CA), anti-IFN-γ (10 μg/ml; Chemicon, Temucula, CA), or anti-IL-12 (Wistar Institute). Supernatants and cells were harvested and analyzed for secreted cytokine or cytokine mRNAs after 24 (for IL-12) or 72 (for IL-10 and IFN-γ) h of culture as described below.

Cytokine ELISA. Supernatants were assayed for IFN-γ, TNF-α, IL-4, and IL-10. IFN-γ was quantitated by a double sandwich ELISA using mouse anti–Hu IFN-γ mAb (Chemicon) and polyclonal rabbit anti–human IFN-γ serum. Hu rIFN-γ (Genentech Inc., San Francisco, CA) was used to generate a standard curve. IL-4 was quantitated in supernatants by a double sandwich ELISA using a mouse anti–human IL-4 mAb (M1) and a polyclonal rabbit anti–human IL-4 sera (P3). Hu rIL-4 (Immunex Corp., Seattle, WA) was used to generate a standard curve ranging from 50 pg/ml to 1 ng/ml. IL-10 was measured using a rat anti–human IL-10 mAb (Cat.# 18551D; PharMingen,) to capture secreted IL-10 and a biotinylated rat anti–human IL-10 mAb (Cat.# 18562D; PharMingen) for detection of bound IL-10 with streptavidin-conjugated horse radish peroxidase and ABTS as substrate. A standard curve was obtained using Hu rIL-10 (kindly provided by DNAX Research Institute, Palo Alto, CA), ranging from 30 pg to 2 ng/ml. IL-12 p40 was measured in cell-free supernatant by RIA (detection limit of 10

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pg/ml) using the mAb pairs C11.79/C8.6 as described (13). Biologically active IL-12 p70 heterodimer (detection limit 1 pg/ml) was measured as described (14).

Cytokine PCR. For cytokine analysis, 0.5–1 ml of PBMC (1–2 × 10^6 cells/ml) were cultured with or without Ag for 48 and 72 h. Supernatants and cells were harvested and analyzed for cytokine mRNAs or secreted cytokines. For cytokine mRNA analysis, total RNA was isolated from PBMC and cDNA synthesized using poly(dT) (Pharmacia, Piscataway, NJ) and AMV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in a final volume of 20 μl. cDNA samples were brought to 200 μl with water. After normalization to β-actin, 12–20 μl of diluted cDNA was amplified by PCR using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) with 0.2 μM of the respective 5′ and 3′ external primers in a reaction volume of 50 μl. The conditions used were: denaturation at 94°C (1 min for β-actin, IL-2, and IL-4; 45 s for IFN-γ; and 30 s for IL-10), annealing at 55°C (1 min for β-actin, IL-2, and IL-4; 30 s for IL-10) or 60°C for 45 s for IFN-γ and elongation at 72°C. We verified that our PCR conditions were within the semi-quantitative range by initially performing serial dilutions of cDNA and varying the number of cycles used for PCR. In all subsequent experiments, 30 cycles were used in the amplification reactions for β-actin, IL-2, IL-4, and IFN-γ. In the case of IL-10 PCR, 25 cycles were used. The primer pairs used were derived from published information: β-actin, IL-2, IL-4, and IFN-γ (15), and IL-10 (16). The nucleotide sequences for the 5′ and 3′ oligonucleotide primers, respectively, are as follows: β-actin, TGACGGGGTCACCCACACTGTGCCCATCTA and CTAGAAGCATTGCGGTGGACGATGGAGGG; IL-2, ATGTACAGGATGCAACTCCTGTCTT and GTCAGTGTTGAGATGATGCTTTGAC; IL-4, ATGGGTCTCACCTCCCAACTGCT and CGAACACTTTGAATATTTCTCTCTCAT; IFN-γ, ATGAAATATACAAGTTATATCTTGGCTTT and GATGCTCTTCGACCTCGAAACAGCAT; IL-10, TCTCAAGGGGCTGGGTCAGCTATCCCA and ATGCCCCAAGCTGAGAACCAAGACCCA.

Inserts were isolated from plasmids containing the human sequences for IL-2, IFN-γ, IL-4, IL-10, and β-actin and radiolabeled ^{32}P-probes generated as described (17). PCR products were analyzed by electrophoresis on 1.5% agarose gels, transferred to nylon membranes, and probed with the appropriate ^{32}P-labeled DNA insert. Hybridizations were at 55°C overnight. Posthybridization washes were at 55°C for 20 min twice each with 2× and 1× SSC containing 0.2% SSC.

Nucleotide Sequence Accession Number. The nucleotide sequence of pLeIF has been submitted to the EMBL/GenBank/DDBJ/Data Bank under accession number U19888.

Results

Molecular Cloning and Sequence Characterization of L. braziliensis Ribosomal eIF4A. As part of a strategy for identifying leishmanial Ag, we screened a *L. braziliensis* genomic expression library with sera from a ML patient, purified immunoreactive rAg, and analyzed them in patient T cell assays for their ability to stimulate proliferative responses and preferential Th1 cytokine production. This led to the identification of several cDNAs. One of these was identified by GenBank sequence comparison as the *L. braziliensis* homologue of the eukaryotic initiation factor 4A (LeIF).

The open reading frame of the full-length LeIF encodes a protein of 403 amino acids and a predicted molecular mass of 45.3 kD (not shown). The lengths (403, 413, 407, and 395 amino acids), molecular mass (45.3, 46.8, 46.4, and 44.7 kD), and isoelectric points (5.9, 5.4, 5.5, and 4.9) of LeIF, and the homologous proteins from tobacco, mouse, and yeast respectively (18–20) are similar. LeIF contains sequence elements characteristic of several demonstrated or putative ATP-dependent RNA helicases represented by the eukaryotic initiation factor 4A (eIF4A). The eIF4A is one of the best characterized members of the "DEAD box" family of RNA helicases. These proteins share a series of conserved motifs, with two of them representing specialized versions of the A and B motifs previously described in other ATP-binding proteins (21). The four amino acid sequence Asp-Glu-Ala-Asp (DEAD) is part of the specialized version of the B motif.

Southern analysis of *L. braziliensis* genomic DNA digested with enzymes that cut both within (PstI and NotI) and outside (BamHI, EcoRI, EcoRV, HindIII, PvuII, and SstI) of LeIF and probed with an ~0.94-kb fragment (nucleotides 143 to 1083) of the coding region of LeIF revealed at least two copies of LeIF (Fig. 1). The same figure also illustrates the cross-species conservation between LeIF of *L. braziliensis* and other *Leishmania* species. Northern analysis revealed that LeIF is transcribed as ~2.5 kb mRNA species (not shown).

Expression, Purification, and Immunoblot Analysis of LeIF Ag. Fig. 2 shows the expression and purification of the ~45 kD LeIF gene product. Immunoblots of lysates from *L. braziliensis* as well as other *Leishmania* species (promastigote and amastigote stages) were performed with the polyclonal rabbit anti-LeIF serum which detected one dominant protein species of ~45 kD. The immune rabbit sera did not react

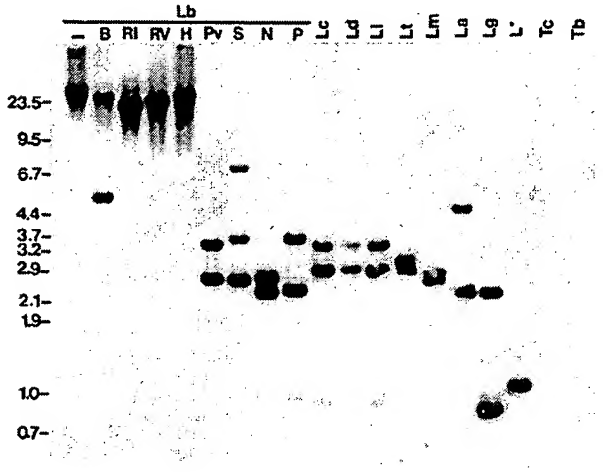

Figure 1. Genomic organization of *Leishmania* eIF4A. *L. braziliensis* DNA (lanes *Lb*, 2.5 μg per lane) was digested with BamHI (lane *B*), EcoRI (lane *RI*), EcoRV (lane *RV*), HindIII (lane *H*), PvuII (lane *Pv*), SstI (lane *S*), NotI (lane *N*), and PstI (lane *P*). DNA from *L. chagasi* (lane *Lc*), *L. donovani* (lane *Ld*), *L. infantum* (lane *Ll*), *L. tropica* (lane *Lt*), *L. major* (lane *Lm*), *L. amazonensis* (lane *La*), *L. guyanensis* (lane *Lg*), an uncharacterized protozoan species (lane *L**), *T. cruzi* (lane *Tc*), and *T. brucei* (lane *Tb*) were digested with PstI. The blot was probed with a ~0.94-kb restriction fragment of LeIF comprising only coding sequences. Numberings indicate the sizes in kb pairs of HindII/HincII–digested λ DNA.

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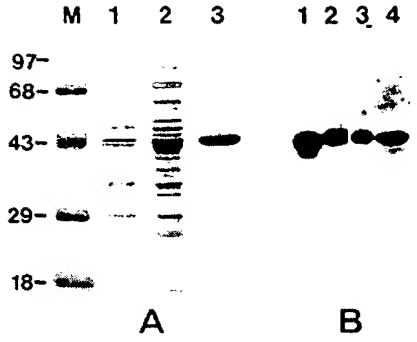

Figure 2. Expression, purification, and immunoblot analysis of LeIF Ag. (*A*) Coomassie blue-stained 12% SDS-PAGE of mol wt markers (lanes *M*), *E. coli* lysates from uninduced (lanes *1*) and induced (lane *2*) cultures and the purified rAg (lanes *3*). (*B*) Reactivities of a rabbit anti-rLeIF serum on *L. braziliensis* and *L. chagasi* promastigote lysates (lanes *1* and *2*, respectively), or *L. amazonensis* promastigote and amastigote stages (lanes *3* and *4*, respectively).

with *E. coli* lysate (not shown), demonstrating the lack of *E. coli* proteins in the purified rLeIF.

rLeIF Stimulates PBMC from Leishmaniasis Patients to Proliferate and to Express a Predominant Th1 Cytokine Profile. Purified rLeIF was tested for its effectiveness in stimulating T cells from *L. braziliensis*–infected individuals. Proliferation and cytokine responses of PBMC from patients with active or healed CL, ML, or individuals with self-healing CL were compared using rLeIF and *L. braziliensis* lysates. PBMC from most (>70%) ML and CL patients proliferated to rLeIF with stimulation indices ranging from 12 to 233 and 2 to 64, respectively (Table 1). PBMC from all six individuals with self-healing CL proliferated in response to rLeIF with stimulation indices (16–198) comparable to those of ML patients. PBMC from two of the self-healing individuals (MS and DJ), proliferated to LeIF to a greater degree than to lysate. Cells from normal individuals were only marginally stimulated by LeIF. The results demonstrate that rLeIF is a potent T cell Ag recognized by a majority of *L. braziliensis*–infected individuals in different stages or manifestations of infection.

A more detailed analysis of cytokine patterns of PBMC from patients with confirmed cases of *L. braziliensis* infection was performed using PCR. Fig. 3 *A* shows the PCR results of cytokine mRNA for three of the six ML patients' PBMC analyzed (JV, SZ, and TE). Cytokine mRNA analyses were performed with freshly isolated PBMC (lanes *0*), or PBMC cultured in the absence (lanes −) or presence of rLeIF. In three of six patients (TE, Fig. 3 *A*; NO and EO, not shown), PBMC not cultured in vitro had detectable levels of mRNA for IFN-γ and IL-4, and two were positive for IL-2 (patients TE and EO). IL-10 mRNA was not detected in uncultured ("resting") PBMC from any of the ML patients. However, after in vitro culture without Ag, IL-10 mRNA was upregulated in most of the ML patient PBMC analyzed. In addition, the levels of other cytokine mRNAs detected in the resting PBMC of patients TE, NO, and EO, decreased to background levels. In three of six patients, leishmanial lysate stimulated the expression of mRNA for the Th1 cytokines (IFN-γ and IL-2) as well as for the Th2 cytokine IL-4. Increased IL-10 mRNA was detected in one of the patients' PBMC (SZ, Fig. 3 *A*) after culture with leishmanial lysate. In contrast, rLeIF Ag elicited increased mRNA for IFN-γ and IL-2 from all ML patient PBMC, an exclusive Th1 cytokine profile. In fact, the addition of LeIF downregulated the production of IL-10 mRNA in the cultured PBMC of most ML patients (Fig. 3 *A*, lanes *eIF*).

Similar PCR analyses were performed on PBMC derived from CL patients. The resting PBMC from three (VS, JP, and CA) of the four patients analyzed revealed readily detectable mRNA for both Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) associated cytokines (Fig. 3 *B*). Little or no mRNA for IFN-γ, IL-2, IL-10, or IL-4 was detected in the resting PBMC of the fourth (AS) CL patient. Therefore, in contrast to ML patients, most patients with CL had RNA for IL-10 as well as for IL-4, IL-2, and IFN-γ. For most patients, whereas the mRNA for IL-2 and IFN-γ were reduced to barely detectable levels after the in vitro culturing of PBMC in the absence of Ag, mRNA for IL-10 remained either unaffected or increased.

All CL patients tested responded to rLeIF or to leishmanial lysate by upregulating the expression of mRNA for IL-2 and IFN-γ. However, IL-4 mRNA was also enhanced in one of the CL patients (AS) after stimulation with LeIF. In three of four patients (JP, VS, and AS), the level of IL-4 mRNA also increased after stimulation with parasite lysate. In the three patients (VS, JP, and CA) with resting levels of IL-10 mRNA, rLeIF downregulated their expression of IL-10 mRNA. We also studied PBMC from a diffuse cutaneous leishmaniasis (DCL) patient (*L. amazonensis* infection). Interestingly, as with PBMC from patients with *L. braziliensis* infection, LeIF downregulated IL-10 mRNA while increasing IFN-γ and IL-2 mRNA in the DCL patient VA (Fig. 3 *B*). PBMC from patient VA also proliferated in the presence of LeIF but not in response to parasite lysate (Table 1). In general, the PCR products for IL-2 and IFN-γ of patient PBMC after stimulation with LeIF or parasite lysate were readily detected on ethidium bromide–stained gels, suggesting relative abundance, whereas IL-4 and IL-10 were detected only by radioactive probing of the resolved PCR products.

The cytokine mRNA profiles of PBMC from self-healing CL were similar to those of ML patients in that (*a*), except for one individual (HS) with detectable levels of IL-10 mRNA, resting PBMC from three of the four patients (GS, AH, DJ, and HS) analyzed had detectable levels of IL-2, IFN-γ, and IL-4 but little or no IL-10 mRNA; (*b*) IL-10 mRNA was upregulated after culture of PBMC without Ag whereas those of IL-2, IFN-γ, and IL-4 decreased to background levels and; (*c*), leishmanial lysate stimulated the expression of a mixed Th1/Th2 cytokine profile whereas LeIF elicited increased mRNA expression of only the Th1-type cytokines and downregulated the expression of IL-10 mRNA in the cultured PBMC of most self-healing CL individuals (not shown).

To complement the cytokine mRNA analysis, we assayed PBMC culture supernatants for IFN-γ, TNF-α, IL-4, and IL-10. Cells from all three patient groups as well as from

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Table 1. *In Vitro Proliferation of PBMC from L. braziliensis-infected Individuals in Response to Parasite Lysate and rLbeIF4A Ag*

Patients	[^{3}H]TdR incorporation (mean cpm [SD] × 10^{-3})				
	Media	Lysate		LeIF	
ML			*SI*		*SI*
JV	0.15 (0.0)	41.30 (1.3)	294	11.90 (4.8)	81
SZ	0.45 (0.1)	140.60 (7.6)	308	105.90 (5.6)	233
AB	0.42 (0.3)	44.20 (0.5)	104	5.00 (1.3)	12
NO	0.38 (0.1)	52.70 (3.3)	138	12.80 (1.6)	33
TE	0.18 (0.0)	27.40 (1.5)	150	8.80 (0.3)	48
MB	0.18 (0.0)	300.10 (9.4)	1634	41.50 (4.5)	226
OM	0.28 (0.0)	35.40 (3.2)	124	6.90 (2.5)	24
CL					
AS	0.22 (0.0)	19.14 (1.3)	87	14.30 (2.3)	64
JP	0.25 (0.0)	55.63 (8.6)	218	4.40 (0.3)	17
VS	0.17 (0.0)	0.26 (0.0)	1.5	0.30 (0.0)	2
RJ	0.10 (0.0)	0.32 (0.2)	3.0	1.50 (0.6)	15
JA	0.16 (0.0)	0.77 (0.1)	4.7	2.50 (0.2)	16
AD	4.20 (1.0)	4.01 (1.0)	2.0	14.10 (2.2)	3.5
HN	0.36 (0.0)	4.73 (1.7)	13	4.69 (1.7)	13
DCL					
VAL	0.22 (0.0)	0.51 (0.3)	2.0	2.12 (0.2)	9.0
SH-CL					
GS	0.21 (0.0)	19.70 (4.4)	94	41.50 (2.8)	198
MS	0.09 (0.0)	0.60 (0.1)	6.5	5.10 (2.1)	57
AH	0.11 (0.0)	59.60 (7.1)	519	9.60 (4.7)	83
DJ	0.12 (0.0)	0.20 (0.1)	1.6	19.00 (6.7)	151
HS	0.12 (0.0)	27.10 (2.0)	225	12.40 (2.7)	103
MCT	0.38 (0.0)	130.30 (14)	340	6.20 (1.5)	16
Normal					
LV	0.14 (0.0)	0.19 (0.0)	1.4	0.71 (0.1)	4.0
VV	0.18 (0.0)	0.31 (0.1)	1.7	0.28 (0.1)	1.5
N3	0.14 (0.0)	0.36 (0.1)	2.6	0.27 (0.1)	1.9
N4	0.59 (0.1)	2.00 (0.3)	3.8	0.56 (0.0)	1.0

a DCL patient (VA) secreted IFN-γ and TNF-α after stimulation with rLeIF (Fig. 4). The levels of both IFN-γ and TNF-α in the supernatants of patient PBMC (0.3–26 ng/ml and 0.1–3 ng/ml, respectively) were significantly higher compared to those from uninfected controls. In the absence of rLeIF stimulation, only PBMC from ML patients (five of six) produced detectable levels of supernatant TNF-α (60–190 pg/ml). Little or no IL-4 or IL-10 were detected in any of the supernatants analyzed (not shown), indicating levels below the detection limit of the ELISA assay employed. By comparison, leishmanial lysate also stimulated patient PBMC to secrete IFN-γ and TNF-α and, in some patients, IL-10 was also detected (not shown). Therefore, and in agreement with the PCR cytokine analysis, whereas the cytokine profile of patient PBMC stimulated with LeIF reflects an exclusive Th1 pattern, parasite lysate stimulates a mixed Th1/Th2 cytokine profile.

LeIF Downregulates the LPS-induced IL-10 Production by Patient PBMC. Taken together, the above results demonstrate that rLeIF elicits a predominant Th1 cytokine profile from the PBMC of ML, CL, or DCL patient while downregulating the expression of IL-10 mRNA. To further demonstrate

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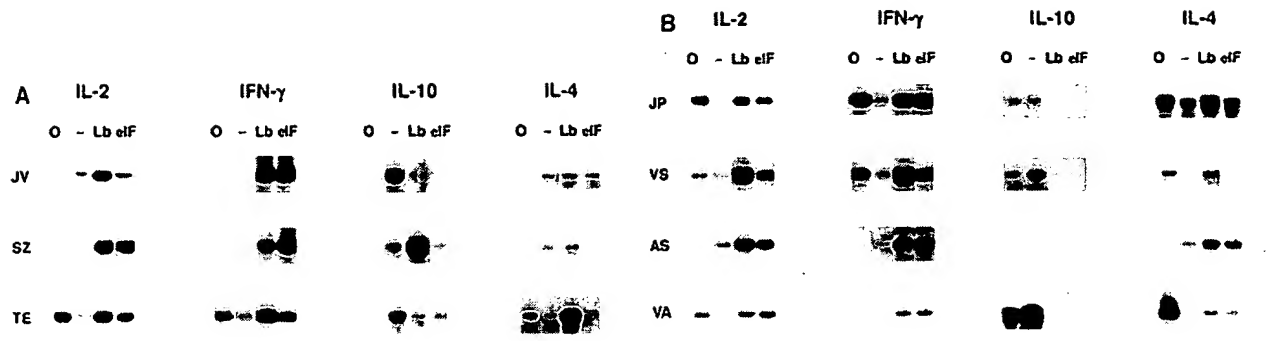

Figure 3. PCR amplification of cytokine mRNAs (*IL2*, *IFN-γ*, *IL10*, and *IL4*) isolated from ML (*A*) and CL (*B*) patients' PBMC before and after stimulation with the indicated Ag. The amount of cDNA synthesis reactions used in the cytokine PCR was normalized to the β-actin PCR product (not shown). (Lanes O and −) PCR products at the initiation of culture or after 72 h of culture in the absence of Ag. PCR products after culturing of PBMC with *L. braziliensis* lysate (lanes *Lb*), and rLeIF (lanes *eIF*) Ag. After hybridization with the respective cytokine probes, the autoradiographs were exposed either at room temperature (*IL2* and *IFN-γ*) for 30 min to 1 h or at −70°C for 6 h to overnight (*IL10* and *IL4*).

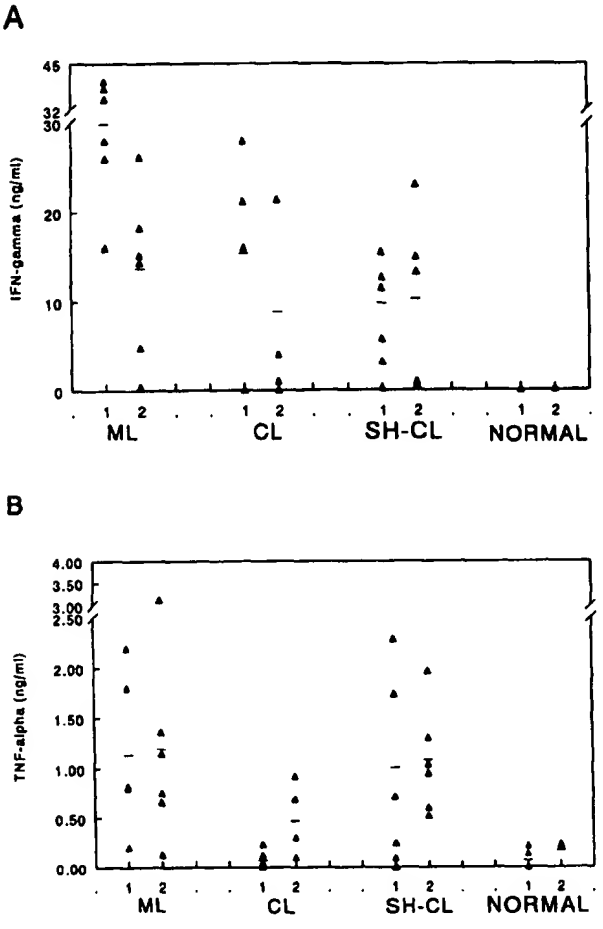

Figure 4. Supernatant levels of IFN-γ (*A*) and TNF-α (*B*) determined by ELISA from 72 h cultures of PBMC from *Leishmania* infected (*ML*, *CL*, and self-healing CL [*SH-CL*]) and control (*NORMAL*) individuals in response to stimulation with parasite lysate (*1*) or rLeIF (*2*). Each value represents an individual patient. Mean values are indicated with a dash for each group.

directly the effect of LeIF in downregulating IL-10 production, PBMC from three ML patients were cultured in the presence of LPS with or without LeIF. LPS stimulated PBMC from all three individuals tested to secrete IL-10 with values ranging from 185 to 424 pg/ml (Fig. 5). However, in the presence of LeIF, the production of LPS-induced IL-10 secretion was reduced by 49–65% (83–166 pg/ml). Because IFN-γ can inhibit IL-10 production by activated macrophages (22) and LeIF stimulated patient PBMC to secrete IFN-γ, the secreted IFN-γ may be responsible for partially inhibiting the production of LPS-induced IL-10.

LeIF Stimulates both Patient and Normal PBMC to Secrete IL-12. IL-12 has been shown to play a pivotal immunoregulatory role in the development of cell-mediated immunity, including the generation of Th1 responses and IFN-γ production in intracellular bacterial or parasitic infections (23). IL-12 is a heterodimeric molecule comprised of p40 and p35 subunits which must be coexpressed for the production of biologically active IL-12 p70 (24). The p40 subunit is produced only by IL-12–producing cells and is induced in vitro and in vivo after bacterial and parasitic stimulation (13, 25), whereas the p35 subunit is both ubiquitous and constitutively expressed. Therefore, cells producing IL-12 p70 also have a large excess (10–100-fold) of biologically inactive free p40 chains (13). Because LeIF stimulated a dominant Th1 cytokine profile and downregulated Th2 responses, we explored a possible role for IL-12 in PBMC responses to rLeIF. Fig. 6 *A* shows that LeIF stimulated IL-12 p40 production from patient PBMC with values consistently higher than those observed with parasite lysate, and that IL-10 abrogated the production of IL-12 p40 by patient PBMC after stimulation with either LeIF or parasite lysate.

PBMC from uninfected individuals also produced IL-12 p40 when cultured with LeIF (Fig. 6 *B*), although no p40 was detected in response to parasite lysate. This may suggest a role for IFN-γ in the lysate-induced p40 observed in patient PBMC, which produced 5–100-fold more IFN-γ than normal PBMC after antigen stimulation (Fig. 4 *A*).

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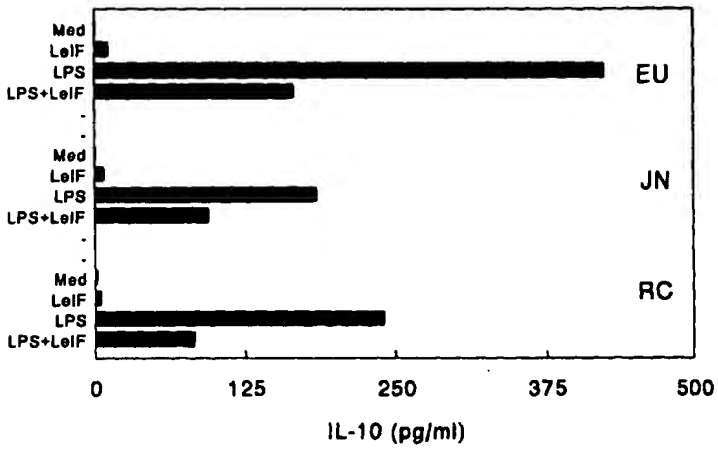

Figure 5. LeIF downregulates the LPS-induced IL-10 production by PBMC. Cells from three ML patients (*EU*, *JN*, and *RC*) were cultured in the absence (*Med*) or presence of LeIF (10 μg/ml), LPS (1 μg/ml) or LPS and LeIF together and the supernatant levels of IL-10 determined after incubation for 3 d.

To determine whether the IL-12 p40 production observed in Ag-stimulated PBMC cultures reflected biologically active cytokine, IL-12 p70 was also determined in these cultures (Fig. 6, *C* and *D*). In general, the p70 production pattern paralleled that of p40, demonstrating that biologically active IL-12 was produced in response to LeIF in both patient and normal PBMC.

Production of IFN-γ by LeIF-stimulated Patient PBMC is IL-12 Dependent and Is Downregulated by IL-10. The above results indicated that the production of IFN-γ by patient PBMC

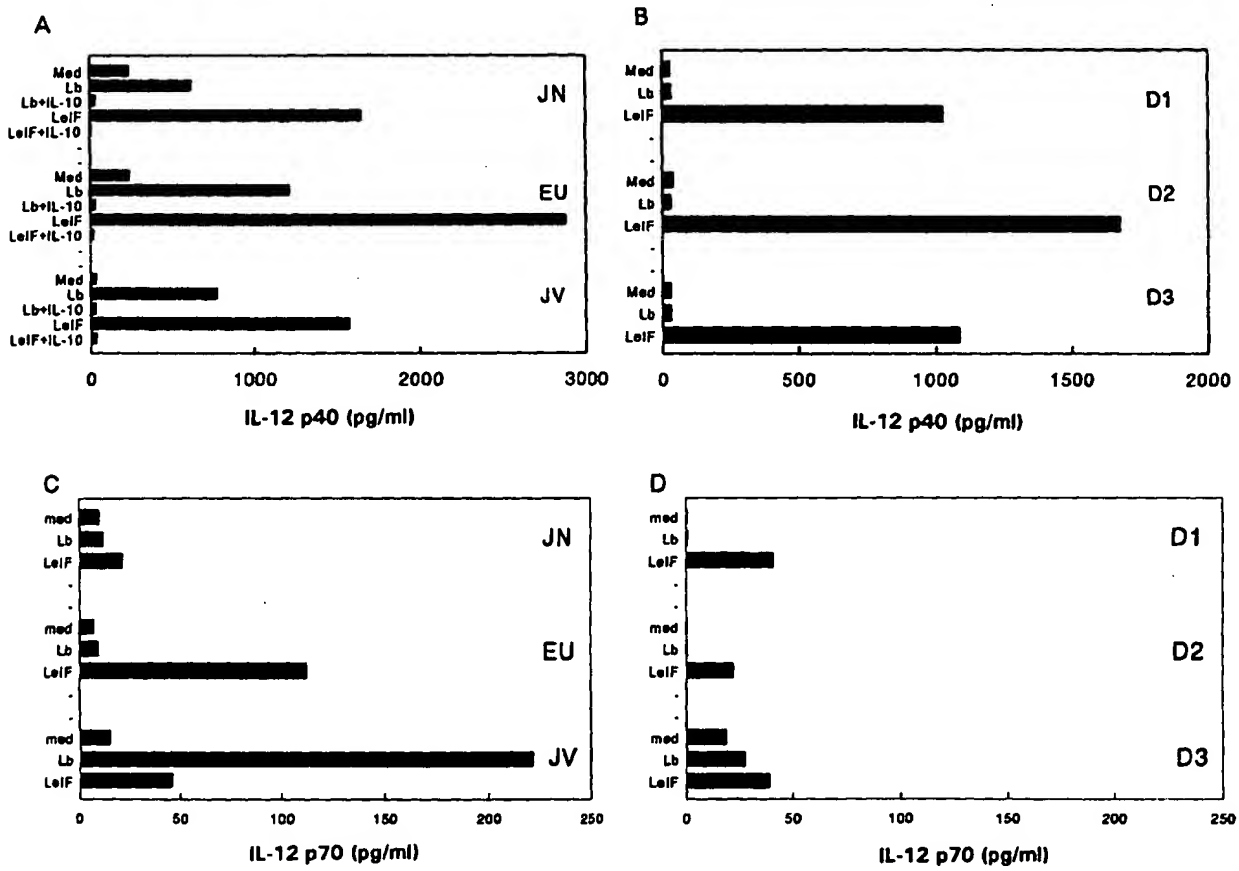

Figure 6. LeIF stimulates PBMC to secrete IL-12 p40 and p70. Cells from (*A* and *C*) ML patients (*JN*, *EU*, and *JV*) and (*B* and *D*) three uninfected individuals (*D1*, *D2*, and *D3*) were cultured in the absence (*Med*) or presence of leishmanial lysate (*Lb*), LeIF, or LeIF and IL-10 and the supernatants from 24-h cultures assayed for secreted IL-12 p40 and p70.

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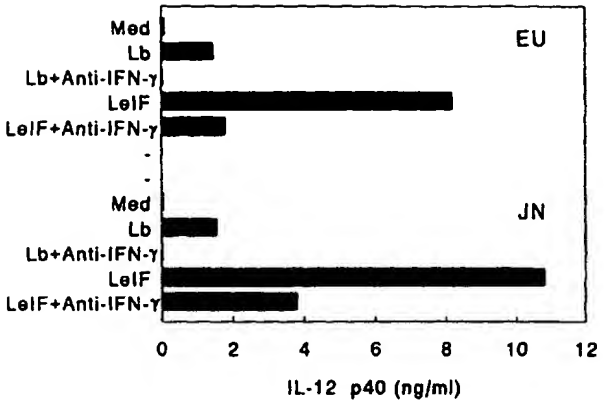

Figure 7. Dependence of IL-12 production on IFN-γ. Supernatants from ML PBMC were assayed for IL-12 p40 in the absence of Ag stimulation (*Med*), in the presence of Ag (*LeIF* and *Lb*) or Ag and anti-IFN-γ mAb.

after stimulation with LeIF or parasite lysate may be mediated by IL-12. LeIF stimulated higher IL-12 p40 in patient PBMC than in normal PBMC indicating that the production of IL-12 may be upregulated by IFN-γ. To determine the interaction between IL-12, IFN-γ, and IL-10 in response to LeIF, PBMC from ML patients were stimulated in vitro with LeIF in the absence or presence of anti-IL-12, anti-IFN-γ mAb, or IL-10, and the cultured supernatants were assayed for IFN-γ and IL-12 p40 secretion. The addition of anti-IFN-γ mAb reduced the LeIF-induced production of IL-12 by 65 and 80% but abrogated the lysate-induced IL-12 (Fig. 7). Both anti-IL-12 mAb and IL-10 abrogated the production of LeIF-induced IFN-γ (Fig. 8 *A*) whereas anti-IL-12 mAb only partially decreased the production of IFN-γ after stimulation with leishmanial lysate (Fig. 8 *B*). Exogenous IL-10 abrogated IL-12 p40 production by patient PBMC in response to either LeIF or lysate (Fig. 8). IL-10 has also been shown to inhibit IL-12 production by human PBMC (26) and by human myeloid leukemia cell lines (14). These results indicate that the production of IFN-γ by LeIF-stimulated patient PBMC is IL-12 dependent, whereas the production of IL-12 is regulated by both IFN-γ–dependent and –independent pathways.

Discussion

We have cloned and characterized rLeIF, a *Leishmania* Ag, that elicits proliferation and a Th1-type cytokine profile from a leishmaniasis patient PBMC. Although resting and lysate stimulated PBMC from *L. braziliensis*–infected individuals have a mixture of Th1- and Th2-like cytokine profiles, rLeIF shifted this pattern towards an exclusive Th1 (IFN-γ and IL-2) cytokine profile. In addition, rLeIF stimulated both patient and normal PBMC to produce IL-12 whereas lysate did not stimulate the production of IL-12 in uninfected individuals. Finally, both rLeIF and parasite lysate stimulated PBMC from ML and self-healing CL patients to secrete TNF-α. The observation that LeIF stimulated T cells from all patients with self-healing CL to proliferate and produce IFN-γ suggests that PBMC responses to LeIF may be associated with protective immunity. In contrast, we have identified other *L. braziliensis* Ag that stimulate patient PBMC to produce a mixed Th1/Th2 or a predominant Th2 cytokine profile or that stimulate PBMC from ML patients but not from self-healing individuals (Skeiky, Y.A.W., et al., manuscript in preparation). Thus, LeIF appears to have unique immunological properties.

Cytokines appear to be essential mediators of immunity to *Leishmania* (27). IFN-γ and TNF-α may synergize to induce leishmanicidal activity in macrophages (28–30). In addition, TNF-α and IL-2 can also synergize with IL-12 to stimulate IFN-γ production by NK cells (31–33). Studies in mice have demonstrated that IL-12 (0.25–1 μg/d) can induce IL-10 production (34, 35). Another study using human monocytes has demonstrated that the addition of TNF-α (5–10 ng) can also induce IL-10 production (36). In our study, TNF-α and IL-12 but not IL-10 were produced by patient PBMC stimulated with LeIF. This may be due to the relatively lower level of either TNF-α or IL-12 produced by pa-

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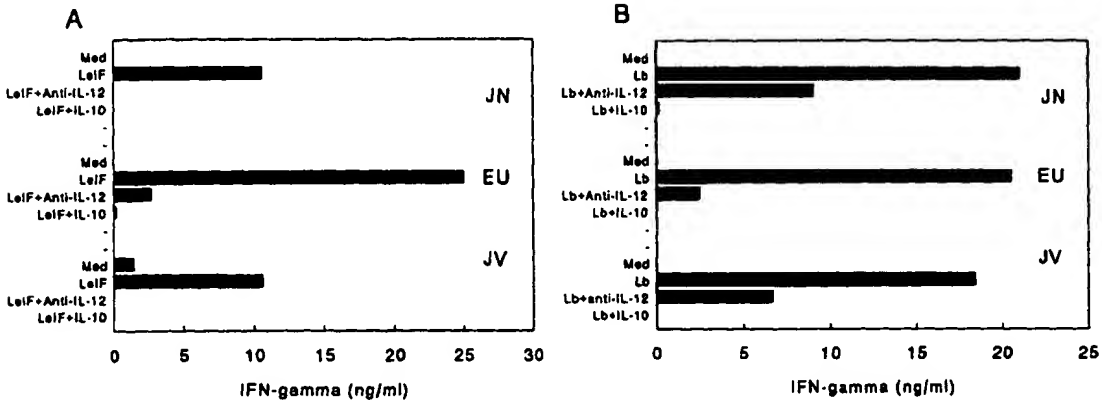

Figure 8. Interdependence of IFN-γ and IL-12 production and their abrogation by IL-10 after stimulation of patient PBMC with LeIF. (*A*) Production of IFN-γ by PBMC from three ML patients (*JN*, *EU*, and *JV*) cultured in the absence (*Med*) or presence of LeIF, LeIF, and anti-IL-12 mAb (*Anti-IL12*) or LeIF and IL-10. (*B*) Same as in *A* but with parasite lysate.

tient PBMC, as well as the ability of IFN-γ, produced in high amounts by patient PBMC, to inhibit the production of IL-10 (22). The use of patient PBMC, rather than purified cell populations, may be more relevant to the in vivo situation since the dominant cytokine pattern is dependent on the interplay of modulatory cytokines.

The presence of a mixture of Th1 and Th2 cytokine mRNAs in the resting PBMC of ML patients is similar to the cytokine profile reported in lesions of ML patients (37, 38), although in our study, IL-10 mRNA was not detected in the resting PBMC from any of the ML patients characterized. However, IL-10 mRNA was detected after in vitro culture of ML patient PBMC in the absence of Ag. In contrast, resting PBMC from CL patients had, in addition to mRNA for IL-4 and IFN-γ, detectable levels of IL-10 mRNA which remained generally unaffected after culture in the absence of Ag. Therefore, the levels of cytokine mRNA in unstimulated controls may reflect an activated state from in vivo exposure to parasite Ag. In this regard, rLeIF downregulated the resting/constitutive levels of IL-10 in patient PBMC's as well as in LPS-stimulated PBMC. It therefore appears that certain *Leishmania* Ag may be able to elicit a dominant Th1 cytokine profile as well as inhibit the production of Th2 cytokines (IL-10) by mechanisms that are not fully understood.

Because IL-12 plays a central role in the initiation and maintenance of Th1 responses in humans and mice (23, 39–43) and is a potent inducer of IFN-γ (33) which can inhibit the production of IL-10 (22), we reasoned that rLeIF may be mediating a dominant Th1 cytokine profile by an IL-12–dependent mechanism. Manetti et al. (41) showed that patients with allergies who have mixed Th1/Th2 cytokine patterns could be shifted towards an experimentally induced Th1 phenotype by the addition of IL-12. A significant finding of the present study was the demonstration that rLeIF stimulated patient PBMC to produce IL-12 and that the production of IFN-γ was, at least in part, IL-12 dependent. Other studies have demonstrated a role for IL-12 in enhancing IFN-γ production in both mouse and human cells (44, 45). Recent studies from several groups have led to the conclusion that the dominant factor of Th1 cell development in response to infection may be determined by the ability of the pathogen to stimulate IL-12 production by macrophages. In this regard, several pathogens including *L. monocytogenes*, *Toxoplasma gondii*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *L. major*, that induce a Th1 response correlate with their capacity to stimulate the production of IL-12 (13, 14, 24, 32, 43, 44, 46, 47). However, the components or factors responsible for stimulating IL-12 production remain to be identified.

Our results have demonstrated that a single component of *Leishmania* is capable of stimulating the production of IL-12. This study represents the first reported identification of a recombinant parasite protein that stimulates the production of IL-12 in both patient and normal PBMC. Therefore, our finding that LeIF can stimulate IL-12 production as well as the elicitation of an exclusive Th1 cytokine profile in patient PBMC indicates that LeIF could confer protective immunity against *Leishmania* infections and/or act as an adjuvant. In human peripheral blood, monocyte/macrophages are the major source of IL-12 although B cells and other minor populations of HLA-DR$^+$ accessory cells also produce IL-12 (13). Our preliminary results indicate the monocytes/macrophages population of patient PBMC as the dominant IL-12–producing cell types after stimulation with LeIF. In addition, LeIF stimulated purified monocytes from uninfected individuals to produce IL-12 but not IL-10, thus suggesting the direct ability of LeIF to function as an adjuvant (Skeiky Y. A. W., M. Kubin, G. Trinchieri, and S. G. Reed, manuscript in preparation). The mechanisms by which LeIF induce IL-12 production are currently being investigated.

In summary, we have identified a *Leishmania* Ag that stimulates IL-12 and elicits a dominant Th1-type cytokine profile in human PBMC. An ideal prophylactic and therapeutic vaccine for leishmanial infection would be one that induces the expansion of Ag-specific Th1 cells. These cells would produce IFN-γ and IL-12 which would promote parasite destruction within the macrophage and downregulate Th2 responses. Because *Leishmania* LeIF induced a powerful Th1-type response, including the two cytokines most clearly associated with protection in experimental leishmaniasis, IFN-γ and IL-12, LeIF is an antigen that merits further study.

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EXR. NOTES

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%%%leishmanial%%% antigen that elicits IL-12 production and Th1-type responses in patients as well as IL-12 production in normal human PBMC.

0009719392 BIOSIS NO.: 199598187225
Cytotoxicity in human mucosal and cutaneous %%%leishmaniasis%%%
AUTHOR: Barral-Netto M (Reprint); Barral Aldina; Brodskyn Claudia; Carvalho E M; Reed S G
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JOURNAL: Parasite Immunology (Oxford) 17 (1): p21-28 1995 %%%1995%%%
ISSN: 0141-9838
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: CD8+ T cells and lysis of parasitized macrophages seem to be important in the resistance to murine %%%leishmaniasis%%%. In the present study, we evaluated peripheral blood mononuclear cell (PBMC) from patients with either cutaneous (CL) or mucosal (ML) %%%leishmaniasis%%% in cell lysis assays using 51-Cr-labeled Daudi or K562 cells, or autologous antigen-pulsed macrophages as targets. Results are reported as lytic units (number of cells required for 30% lysis) per million PBMC. Exposure of patient PBMC (n = 12) to %%%lysate%%% from %%%Leishmania%%% amazonensis promastigotes led to an increase in cytotoxic activity compared to unstimulated patient cells against Daudi (81.8 +- 14.9 vs 13.6 +- 5 lytic units (LU) per million PBMC; mean +- SEM) and K562 (65.7 +- 8.4 vs 13.1 +- 5 LU/10-6 PBMC). ML had higher responses when CL in

0008772404 BIOSIS NO.: 199395074670
Mapping human T cell epitopes in %%%Leishmania%%% gp63: Identification of cross-reactive and species-specific epitopes
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JOURNAL: Journal of Immunology 150 (3): p932-939 %%%1993%%%
ISSN: 0022-1767
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

0008292116 BIOSIS NO.: 199293135007
A RIBONUCLEASE ACTIVITY IS ACTIVATED BY HEPARIN OR BY DIGESTION WITH PROTEINASE K IN MITOCHONDRIAL EXTRACTS OF %%%LEISHMANIA%%%-TARENTOLAE
AUTHOR: SIMPSON A M (Reprint); BAKALARA N; SIMPSON L
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JOURNAL: Journal of Biological Chemistry 267 (10): p6782-6788 %%%1992%%%
ISSN: 0021-9258
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: A ribonuclease activity in a 100,000 .times. g supernatant of a Triton %%%lysate%%% of a mitochondrial-kinetoplast fraction from %%%Leishmania%%% tarentolae is activated by incubation with heparin or by predigestion of the %%%lysate%%% with proteinase k or pronase. In vitro-transcribed preedited cytochrome b mRNA is cleaved at several

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A Ribonuclease Activity Is Activated by Heparin or by Digestion with Proteinase K in Mitochondrial Extracts of *Leishmania tarentolae**

(Received for publication, November 5, 1991)

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A ribonuclease activity in a 100,000 × *g* supernatant of a Triton lysate of a mitochondrial-kinetoplast fraction from *Leishmania tarentolae* is activated by incubation with heparin or by predigestion of the lysate with proteinase k or pronase. *In vitro*-transcribed pre-edited cytochrome *b* mRNA is cleaved at several sites. With time, complete degradation of the RNA occurs. All cleavages occurred within putative single-stranded regions of the RNA. No cleavage was observed with 9 S rRNA. The presence of a nonspecific nucleotide or nucleoside slows the rate of cleavage. The cleavage activity is inhibited by sodium dodecyl sulfate or phenol/chloroform extraction, is retained by a 10-kDa cutoff filter, and passes through a 30-kDa filter. Micrococcal nuclease inhibits the proteinase-induced activity but not the heparin-induced activity.

Several ribonuclease activities have been shown to exist in mitochondria. An RNase P activity has been identified in HeLa mitochondria which cleaves the precursor to *Escherichia coli* $tRNA^{Tyr}$ at the same site as the *E. coli* RNase P, producing a mature 5′-end of the tRNA (1). It is thought that the HeLa mitochondrial RNase P is involved *in vivo* with mitochondrial tRNA processing. In mouse mitochondria, an RNA-processing endoribonuclease (RNase MRP) has been identified which cleaves mitochondrial RNA complementary to the light strand at a unique site and is thought to be involved in the initiation of mitochondrial DNA replication (2). The 136-nt[1] RNA associated with this enzyme is a nuclear gene product also found associated with the nucleolus (3–5). The mitochondrial MRP and mammalian RNase P share a common antigenic epitope (6), but it has been shown that RNase MRP and HeLa mitochondrial RNase P are different enzymes.[2] In yeast, an RNase P activity which is involved in 5′ processing of mitochondrial tRNAs has been demonstrated in purified mitochondria (7). This enzyme has an RNA component which is encoded at the tRNA synthesis locus in *Saccharomyces cerevisiae* (8) and a homologous locus in *Candida glabrata* (9). Another endoribonuclease activity has been detected in yeast mitochondria which removes the 3′ trailer sequences from 5′-matured tRNA precursors (10).

In the kinetoplast-mitochondrion of the kinetoplast protozoa, nothing is known about the ribonucleases which almost certainly are involved with the processing and turnover of mitochondrial rRNAs, mRNAs, and guide RNAs (gRNAs) transcribed from the maxicircle and minicircle DNA molecules (11). The post-transcriptional RNA modification system known as RNA editing, which inserts and deletes uridine (U) residues from mRNAs, may also require the assistance of specific endo- and exoribonucleases (12–15). Finally, the apparent tRNA importation system may require specific nucleases for RNA processing (16, 17).

In this paper, we demonstrate the presence of a cryptic RNA cleavage activity in mitochondrial extracts from *Leishmania tarentolae*, which is activated by treatment with heparin or by digestion with proteinase K, and discuss the possible role of this activity in RNA processing.

EXPERIMENTAL PROCEDURES

Cell Culture and Mitochondrial Isolation—*L. tarentolae* cells (UC strain) were grown in brain heart infusion medium (Difco) at 27 °C as described previously (18). The mitochondrial-kinetoplast fraction was isolated from late log phase cells (100–180 × 10^6 cells/ml) by flotation in Renografin density gradients after cell rupture under hypotonic conditions as described previously (18, 19). The final washed mitochondrial pellet was resuspended at 5 mg of protein/ml in 20 mM Hepes (pH 7.5), 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, and stored frozen in 200-μl aliquots at −80 °C.

Preparation of Mitochondrial Extracts—The thawed mitochondrial suspension (200 μl) was homogenized for 15 s at 5 °C with 0.3% Triton X-100 (Pierce) in a Microfuge tube (Kontes, Pellet Pestle Mixer, motor-driven), yielding the TL extract (20). Centrifugation of TL for 30 min in a Microfuge at 12,000 × *g* yielded the TS extract (20). Centrifugation of TS for 100,000 × *g* for 60 min yielded the S-100 extract. Proteinase K (Bethesda Research Laboratories) was dissolved at 10 mg/ml in 50 mM Tris-HCl (pH 8), 1 mM $CaCl_2$. Predigestion of the TL or the TS extract was performed by addition of 100 μg/ml proteinase K and incubation for 5 min at 37 °C.

In Vitro Transcription—A 198-base pair *AccI/RsaI* restriction fragment from the pLt120 maxicircle region (nucleotides 5347–5545 of the *L. tarentolae* maxicircle sequence; entry LEIKPMAX in GenBank) was cloned into the *SmaI* site of the pBluescript SK (−) vector (Stratagene) to yield the pNB2 plasmid. pNB2 plasmid DNA digested with *BamHI* was used as template to synthesize RNA using T7 polymerase, as described previously (20). For uniformly labeled (UL) RNA, the concentration of cold UTP was decreased 3-fold and 10 μCi of [^{32}P]UTP (800 Ci/mmol) was added. The 272-nt pNB2 RNA (5′ to 3′) contained a 73-nt vector sequence at the 5′ end followed by 199-nt maxicircle sequence. This sequence represented the 5′ portion of the cytochrome *b* (CYb) gene, containing the 22-nt pre-edited region (PER) with a 56-nt sequence 5′ flanking and 186-nt sequence 3′ flanking. RNA was recovered from the preparative acrylamide gel by diffusion and ethanol precipitation.

The 9 S rRNA coding sequence (21) was PCR-amplified from the

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[1] The abbreviations used are: nt, nucleotide(s); gRNA, guide RNA; CYb, cytochrome *b*; TUTase, terminal uridylyltransferase; TL, Triton lysate; TS, Triton supernatant; S-100, 100,000 × *g*-1 h supernatant of TS; PER, pre-edited region; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; AMP-PNP, adenyl-5′-yl imidodiphosphate.

[2] G. Attardi, personal communication.

pLt120 cloned maxicircle DNA (nt 1639-2249 in LEIKPMAX); the 5′ PCR primer contained a T7 promoter sequence. *In vitro* transcription of the PCR product with T7 polymerase yielded the 611-nt 9 S rRNA, which was used as a control in the cleavage experiments.

Cleavage Reaction—The reaction mixture contained 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, approximately 10^4 cpm of uniformly labeled (UL) RNA (10^6 cpm/μg), and 5 μg/ml heparin (Sigma). Other nucleotides or nucleosides, when substituted for ATP, were used at 1 mM. Mitochondrial extract (TL, TS, or S-100) (10 μl/50 μl reaction volume) was added to start the reaction. After a 1-h incubation at 27 °C, the reaction was terminated by extraction with phenol/chloroform, and the RNA was analyzed in an 8% acrylamide, 7 M urea gel. The gel was fixed and dried for autoradiography.

Micrococcal Nuclease and Mung Bean Nuclease Digestion—Micrococcal nuclease (Pharmacia LKB Biotechnology Inc.) stock solution was 8 units/μl in 10 mM Tris-HCl (pH 8), 1 mM $CaCl_2$. Digestion of TL or TS extract was performed by addition of 1 mM $CaCl_2$ and 16 units of micrococcal nuclease to a 50-μl extract and incubation for 30 min at 4 °C. The reaction was terminated by addition of 2.5 mM EGTA.

Limiting digestion of pNB2 RNA with 3.2×10^{-4} units of micrococcal nuclease was performed in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 4 mM $CaCl_2$, for 30 min at 4 °C. Limiting digestion of pNB2 RNA with 1.6×10^{-3} units/μl mung bean nuclease (Bethesda Research Laboratories) was performed in 30 mM sodium acetate (pH 5), 50 mM NaCl, 1 mM zinc acetate, 5% glycerol, 50 μg/ml bovine serum albumin, for 20 min at 27 °C.

Centrifugal Filtration of S-100 Mitochondrial Extract—The TL extract was predigested with proteinase K and centrifuged at 100,000 × *g* for 1 h to obtain the S-100 extract. This extract was subjected to centrifugal filtration (2000 × *g* for 40-90 min) through Millipore Ultrafree-MC 10,000 NMWL or 30,000 NMWL filters. The filtrate was assayed for cleavage activity in the presence of heparin using pNB2 RNA as substrate.

RNA Sequencing—The pNB2 RNA cleavage products were recovered from a preparative gel and used as template for dideoxy chain termination sequencing as described previously (12). The sequencing primer (5′-CCTAAACTAAACCTACACC-3′) (nt 5475-5456 in GenBank entry LEIKPMAX) was 5′-end-labeled with [γ-^{32}P]ATP (>7000 Ci/mmol; ICN) and T4 polynucleotide kinase (Bethesda Research Laboratories).

Computer Analysis—The pNB2 RNA sequence was analyzed for secondary structures by the FOLD program of Zuker and Stiegler (22).

RESULTS

Presence of Heparin Induces Ribonuclease Activity in Mitochondrial Extract—T7-synthesized pre-edited cytochrome *b* mRNA (pNB2 RNA) is not degraded by 1-h incubation at 27 °C with unclarified or clarified mitochondrial Triton extracts (TL or TS extracts). A sonicated mitochondrial extract without Triton also showed an absence of ribonuclease activity (data not shown). pNB2 RNA was, however, extensively labeled under TUTase conditions (see "Experimental Procedures") in the presence of TL extract with [^{32}P]UTP without any degradation. However, if heparin was present to inhibit the activity of the TUTase (20), UL pNB2 RNA was degraded into several specific fragments after the standard 40-min incubation at 27 °C, suggesting that a nuclease was activated which cleaved the RNA at several specific sites (Fig. 1*A*). This was surprising since heparin has not been previously reported to stimulate RNase activity.

The standard pNB2 RNA cleavage reaction consisted of the addition of 1/5 volume of TL, TS, or S-100 extract to pNB2 RNA in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP for 1 h at 27 °C. The addition of heparin (5 μg/ml) was required to activate the cleavage activity. The RNA cleavage products were analyzed in an 8% acrylamide, 7 M urea gel. The ATP requirement will be discussed below.

Predigestion of the Mitochondrial Lysate with Proteinase K or Pronase Also Induces Ribonuclease Activity—An identical pNB2 RNA cleavage pattern was obtained in the absence of heparin by a brief predigestion of the TL extract with proteinase K (Fig. 1*A*); addition of heparin during the reaction was synergistic. The effectiveness of the protease digestion in degrading protein was confirmed by SDS gel analysis (data not shown). Predigestion with another broad spectrum protease, pronase B, also induced this specific cleavage activity (Fig. 1*B*). Activation of the cleavage activity also occurred when the Microfuge-clarified extract (TS extract) was extracted with StrataClean (Stratagene) resin (data not shown). This silica-based resin contains hydroxyl groups which interact with proteins in a manner similar to the hydroxyl groups of phenol and thereby remove the proteins from solution.

However, deproteinization with phenol/chloroform or addition of 0.2% sodium dodecyl sulfate both effectively inhib-

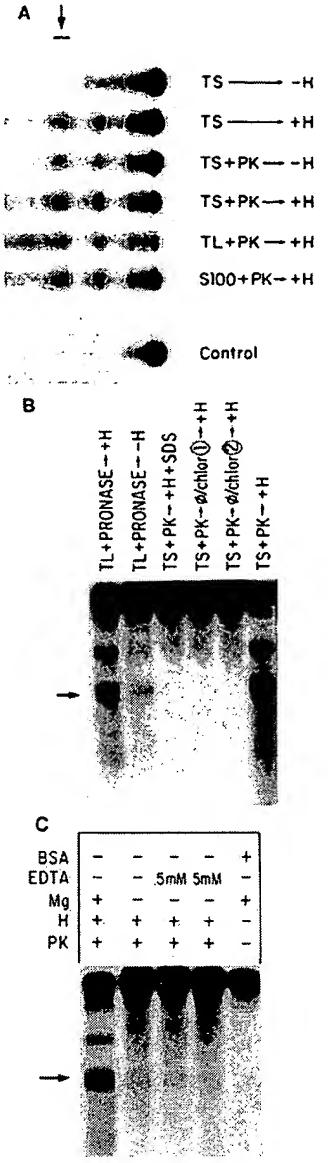

FIG. 1. **Activation of cleavage of pNB2 RNA by addition of heparin to TL or TS extract or by predigestion with proteinase K or pronase.** Uniformly labeled (UL) pNB2 RNA was incubated with TS, TL, or S-100 extract as indicated above each lane. *−H*, no heparin added; *+H*, heparin added; *PK*, proteinase K predigestion; *Control*, no incubation. The *bracket* and *arrow* indicate the two major products resulting from cleavage within the PER. *A*, activation by heparin or proteinase K digestion. *B*, effect of pronase, SDS, or phenol/chloroform treatment on cleavage activity. *C*, Mg^{2+} requirement of cleavage activity.

ited the proteinase K or heparin-activated cleavage activity, as shown in Fig. 1*B*. These data suggest that the cleavage activity is probably due to a protease-resistant protein and not to a catalytic RNA. Fig. 1 also shows that Microfuge centrifugation of the TL extract to produce the TS extract or high speed centrifugation to produce the S-100 extract have no effect on the proteinase K + heparin-inducible cleavage activity, suggesting that the induced activity is not membrane-bound. Proteinase K digestion of S-100 extract also induces the activity (data not shown), indicating that the activity is in a soluble form prior to protease digestion.

Substitution of bovine serum albumin for the proteinase K in the predigestion of the TL extract did not activate the cleavage activity (Fig. 1*C*), indicating that the activation by proteinase K was not a nonspecific phenomenon caused by protein binding of some cofactor.

The presence of Mg^{2+} was required for proteinase K + heparin-induced cleavage activity. As shown in Fig. 1*C*, deletion of $MgCl_2$ from the reaction mix and removal of endogenous Mg^{2+} by the addition of EDTA inhibited the reaction.

The proteinase K + heparin-induced activity of the TL extract did not affect pNB2 plasmid DNA in a specific manner, but a nonspecific DNase activity was observed in the TL extract, which was inhibited by the addition of heparin.

Major Cleavage Sites of pNB2 RNA Are within the Pre-edited Region—The two approximately 140-nt major cleavage products indicated by a bracket in Fig. 2*A* were gel-isolated and subjected to sequence analysis using a primer downstream of the pre-edited region. As shown in the sequencing gel of Fig. 2*B*, a strong termination in all lanes occurred between 2 G residues, as indicated by the nucleotide labeled N·. This was interpreted as being due to truncated molecules produced by a specific cleavage. Four other less frequent cleavages also occurred in this region. Also indicated in this figure on the left side are the location of sites of addition of U residues in the mature edited mRNA. It may prove relevant that the major cleavage site is located 2 nt upstream of the first expected editing site (23) and that the four minor cleavages are also located within the pre-edited region (PER).

The other cleavage sites giving rise to the three higher molecular weight bands were not precisely mapped, but from the sizes (220 nt, 190 nt, 180 nt) of the fragments and their specific 5′ end-labeling with [γ-^{32}P]ATP and T4 kinase, these sites are localized outside the PER (data not shown). The relative amounts of these three cleavage products as compared to the 140-nt products varied between experiments (compare the patterns in Figs. 1*A* and 2*A* with those in Figs. 4 and 5), but conditions can be obtained in which the 140-nt bands represent the major cleavage products.

The Proteinase K + Heparin-induced Nuclease Activity Does Not Affect the Mitochondrial 9 S rRNA—Mitochondrial 9 S rRNA is apparently not internally or 5′-edited *in vivo* (24). T7-synthesized 9 S RNA was unaffected by incubation for 1 h at 27 °C in the presence of proteinase K + heparin-treated TL extract under conditions in which pNB2 RNA is cleaved (data not shown).

Ultrafiltration of the Cleavage Activity Indicates a Molecular Size between 10 kDa and 30 kDa—As shown in Fig. 3, the proteinase K + heparin-activated pNB2 RNA cleavage activity was retained by a 10-kDa cutoff centrifugal filter (Millipore) and passed through a 30-kDa cutoff filter. This suggests that the cleavage activity consists of one or more components that have a molecular size between 10 and 30 kDa.

Presence of Nucleotide Affects Rate of Cleavage Reaction—Removal of ATP from the standard TL reaction mixture resulted in complete degradation of pNB2 RNA to small fragments after a 1-h incubation at 27 °C (Fig. 4). However, ADP, AMP or a nonhydrolyzable analogue of ATP, AMP-PNP, each could substitute for ATP in producing the characteristic cleavage pattern (Fig. 4*A*). Other nucleotides had varying levels of effectiveness in substituting for ATP. As shown in Fig. 4*B*, AMP or dATP yielded a similar cleavage pattern as ATP, CTP and UTP had little effect, and GTP almost completely inhibited the cleavage under standard reaction conditions. Adenosine was as effective as ATP (data not shown). The nucleotide effect was apparently on the

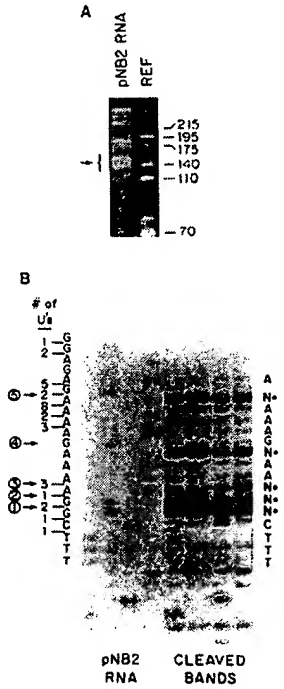

FIG. 2. **Localization of sites of cleavage within PER.** *A*, preparative gel of cleaved pNB2 RNA for sequence analysis. The *bracket* and *arrow* indicate the two bands that were gel-isolated. *REF*, *L. tarentolae* cytoplasmic rRNA and tRNA molecular weight markers. *B*, sequencing ladders of pNB2 RNA and the cleavage products from *A*. The sites and number of U residues added in editing are indicated on the *right*. The cleavage sites are indicated by *arrows* and by N· on the *right*.

FIG. 3. **Centrifugal filtration of the cleavage activity.** TL extract was digested with PK and then clarified at 100,000 × *g* for 1 h. The resulting S-100 supernatant was subjected to centrifugal filtration through a Millipore 10-kDa-cutoff filter or a 30-kDa cutoff filter. Each filtrate was tested for cleavage activity using UL pNB2 RNA as shown.

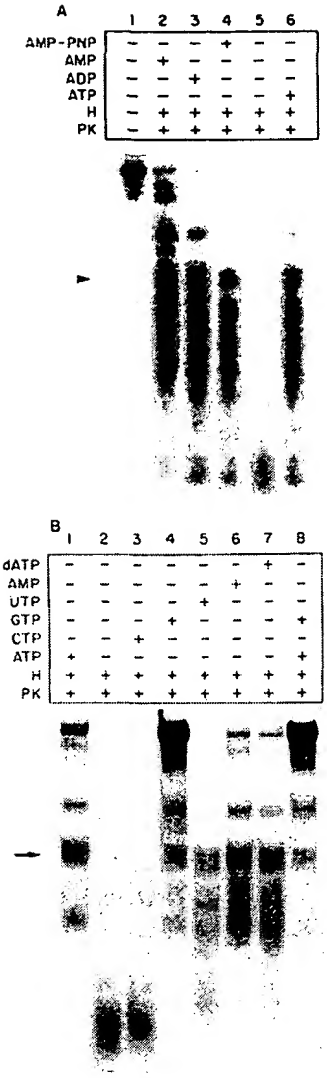

FIG. 4. **Nucleotide requirement of cleavage activity.** UL pNB2 RNA was treated with TL extract under standard cleavage conditions. *PK*, proteinase K-predigested; *H*, heparin added to cleavage reaction mixture. *A*, the presence or absence of ATP, ADP, AMP, or AMP-PNP is indicated above each lane. The *arrow* indicates the localization of the two products produced by cleavage within the PER. *B*, substitution of CTP, GTP, UTP, AMP, and dATP for ATP.

kinetics of cleavage, as GTP at 37 °C gave a similar pattern as ATP at 27 °C, whereas ATP at 37 °C produced complete degradation and at 4 °C inhibited cleavage completely (Fig. 5*A*). This was confirmed by the experiment in Fig. 5*B*, in which incubation of pNB2 RNA in TL extract at 4 °C in the absence of any added nucleotide yielded the identical cleavage pattern as with ATP at 27 °C.

The 3′ OH of the Nucleotide Cofactor Is Not Required for Inhibition of Cleavage Activity—Both cordycepin (3′-deoxy-ATP) and 2′,3′-dideoxy-ATP could substitute for ATP in the standard pNB2 RNA cleavage reaction, suggesting that the 3′ OH of the added nucleotide is not required (Fig. 6). In addition, [α-^{32}P]-ATP was not covalently linked to the cleaved fragments after the reaction (data not shown). This evidence and that presented above clearly showed that the nucleotide requirement was not due to a 3′ OH nucleophilic attack and transesterification reaction analogous to the role of guanosine or GMP in group I splicing (25).

Micrococcal Nuclease Prevents the Activation of the Cleavage Activity by Proteinase K Digestion but Not by Heparin—Digestion of the proteinase K-digested TS extract with micrococcal nuclease completely inhibited the proteinase K-induced pNB2 RNA cleavage activity (Fig. 7*A*). However, the inhibitory effect of micrococcal nuclease on the proteinase K-induced cleavage activity was also observed when the micro-

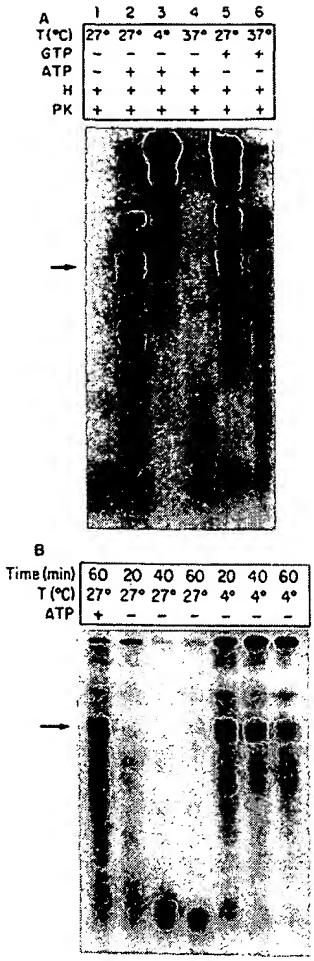

FIG. 5. **Effect of temperature of incubation on cleavage of pNB2 RNA in presence of ATP or GTP.** UL pNB2 RNA was treated in the presence of heparin and either ATP or GTP with proteinase K (*PK*)-predigested TL for the indicated times at the indicated temperatures. *A*, incubation at 37 °C with GTP produces the same result as incubation at 27 °C with ATP. *B*, cleavage reaction in absence of ATP at 4 °C resembles that in presence of ATP at 27 °C.

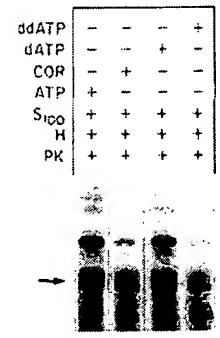

FIG. 6. **The 3′-OH is not required for the ATP effect on the cleavage reaction.** Details are shown above each lane. *COR*, cordecypin.

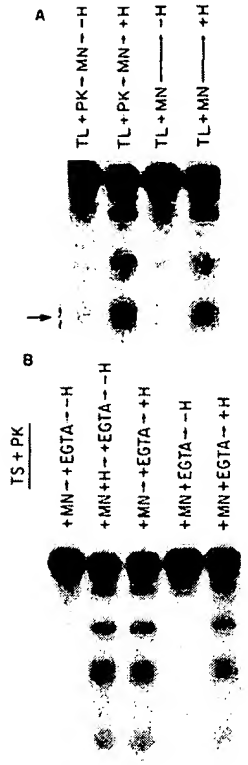

FIG. 7. **Micrococcal nuclease inhibits proteinase K-activated nuclease activity and heparin restores activity.** Details are shown above each lane. *MN*, micrococcal nuclease digestion. The micrococcal nuclease digestion was terminated by addition of 2.5 mM EGTA. *−H* or *+H*, cleavage reaction performed in absence or presence of heparin. *A*, effect of micrococcal nuclease digestion on induction of cleavage activity by proteinase K (*PK*). *B*, EGTA-inhibited micrococcal nuclease also inhibits the proteinase K-induced cleavage activity.

coccal nuclease digestion was performed in the presence of EGTA, which inhibits the nuclease activity of micrococcal nuclease by removal of Ca^{2+} (Fig. 7*B*), indicating that the micrococcal nuclease inhibition effect was not due to degradation of an RNA component.

Addition of heparin during the cleavage reaction or during the micrococcal nuclease predigestion reactivated the micrococcal nuclease-inhibited cleavage activity (Fig. 7, *A* and *B*). Gel analysis of the treated lysate showed that micrococcal nuclease digestion (in the absence of EGTA) was effective in degrading RNA, including gRNA, and that the addition of EGTA completely inhibited the digestion of RNA (data not shown). Control experiments also demonstrated that the presence of the proteinase K during the 30-min treatment with micrococcal nuclease at 5 °C did not greatly affect the nuclease activity of the micrococcal nuclease itself (data not shown).

This evidence indicates that micrococcal nuclease is inhibiting the PK-induced pNB2 RNA cleavage activity by binding a cofactor (26) rather than by degrading RNA. Heparin would then release the bound cofactor.

Limiting Digestion of pNB2 RNA with Mung Bean Nuclease and Micrococcal Nuclease—UL pNB2 RNA was digested with limiting amounts of mung bean nuclease and micrococcal nuclease, and the digestion patterns compared with that produced by digestion with the proteinase K + heparin-activated mitochondrial cleavage activity (Fig. 8). A discrete series of bands is produced by limiting micrococcal nuclease and mung bean nuclease digestion, indicating the presence of labile regions in the intact pNB2 RNA molecule. The mung bean

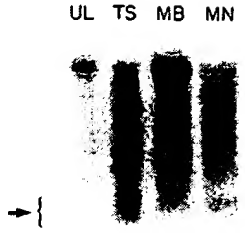

FIG. 8. **Comparison of the patterns produced by limiting digestion of pNB2 RNA with micrococcal nuclease and mung bean nuclease with the proteinase K + heparin-activated cleavage pattern.** *UL*, UL pNB2 RNA untreated control. *TS*, UL pNB2 RNA digested with proteinase K + heparin-treated TS for 1 h at 27 °C. *MB*, UL pNB2 RNA digested with limiting mung bean nuclease. *MN*, UL pNB2 RNA digested with limiting micrococcal nuclease. *Arrow* indicates two fragments produced by cleavage within PER in the TS lane.

nuclease gives rise to four bands running at approximately the same position as the upper four bands obtained with proteinase K + heparin cleavage. Three of the micrococcal nuclease bands also co-migrate with proteinase K + heparin bands. However, the two prominent cleavage fragments in the proteinase K + heparin-digested RNA which are produced by a major cleavage just upstream of editing site 1 correspond in the micrococcal nuclease or mung bean-digested RNA to a single band, suggesting that the sites of cleavage are not identical.

DISCUSSION

This investigation was stimulated by the observation that synthetic labeled pre-edited CYb RNA (pNB2 RNA) is not degraded by incubation for 1 h at 27 °C in the presence of the mitochondrial TL extract under conditions in which the mitochondrial TUTase (20) adds multiple 3′ terminal U residues. However, if the TUTase activity is inhibited by the addition of 5 μg/ml heparin, the exogenous pNB2 RNA is cleaved into several specific fragments during this incubation. Heparin is apparently activating a latent ribonuclease which has some specificity. The fact that the identical cleavage pattern occurred if the TL extract was preincubated with a broad spectrum protease such as proteinase K or pronase suggests that the same ribonuclease activity can be activated in several ways and may be due to the same enzyme. However, it is possible that different ribonucleases are activated by proteinase K digestion or by heparin which have the same substrate specificity. The pNB2 cleavages all occur within putative single-stranded regions, as determined by the FOLD program (Fig. 9). A major early cleavage site was precisely mapped by sequencing cleaved fragments to be 2 nt upstream from the first expected site of uridine addition in the mature edited RNA and a cluster of four other cleavages were all within the pre-edited region. With longer incubation periods, several additional cleavages occurred at other sites in the pNB2 RNA, eventually producing complete degradation.

The nonspecific requirement of a nucleotide for the accumulation of the major cleaved fragments at room temperature appears to be due to an inhibitory effect on the reaction which slows the kinetics of the fragmentation reaction at 27 °C and allows accumulation of the two specific fragments resulting from cleavage within the PER. Continued incubation results in multiple additional cleavages and complete fragmentation of the RNA. The mechanism of this inhibition is not understood, but it does not involve hydrolysis of the nucleotide nor require a 3′-OH group. We suggest that the nucleotide effect may be indirect due to the fact that this is a crude extract.

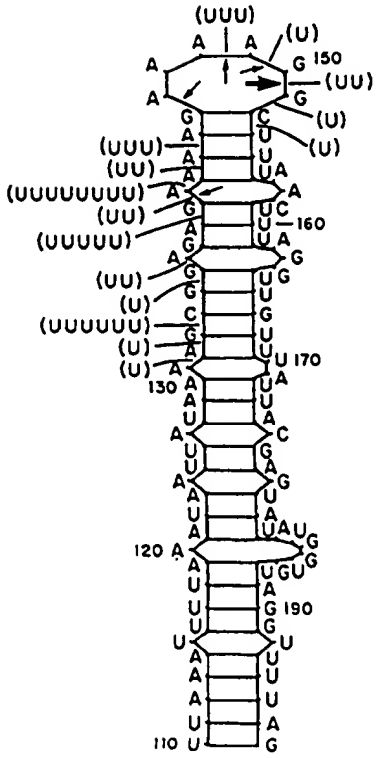

FIG. 9. **Secondary structure of pNB2 RNA predicted by FOLD program.** The number and location of U residues added by editing in the mature edited mRNA are indicated. The *dark arrow* indicates the major cleavage site and the *light arrows* the minor cleavage sites within the PER.

The molecular nature of the proteinase K + heparin-activated ribonuclease is still obscure. The resistance to broad spectrum proteolytic enzymes would appear to suggest the presence of a catalytic RNA component, similar to the known mitochondrial RNase P (1, 8) or MRP (6) enzymes. However, the sensitivity to SDS and to deproteinization by other means and the resistance of the heparin-activated activity to micrococcal nuclease digestion suggest a protein-based activity. The apparent single strand specificity of the heparin-activated activity suggests a possible relationship to a nonspecific single strand ribonuclease. The presence of nuclease-sensitive regions in the native pNB2 RNA molecule was confirmed by limiting digestions with mung bean nuclease and micrococcal nuclease. However, the fact that the fragment patterns and stoichiometries produced by these two activities are not identical indicates that the mitochondrial cleavage activity may possess additional specificities for recognition of cleavage sites other than the recognition of single-stranded regions.

The function of the proteinase K + heparin-activated ribonuclease in mitochondrial RNA metabolism is not known. RNA turnover has been shown to represent an important control mechanism controlling the steady state levels of the 9 S and 12 S rRNAs during the African trypanosome life cycle (27). One possible function of the kinetoplast ribonuclease is that this activity represents an RNA degradation enzyme involved *in vivo* with RNA turnover, which has some specificity for single-stranded regions. However, it is hard to understand why this activity would be activated by such divergent methods as heparin incubation or protease digestion.

RNA processing represents another possible role of the mitochondrial ribonuclease. There is some evidence for the existence of high molecular weight precursors for maxicircle transcripts which must be processed to yield the mature species (28–30).

Another possible function is in the process of RNA editing of maxicircle mRNAs. Two models for editing have been proposed (13, 14), and, in both models, a cleavage activity is invoked. However, to explain the activation of the cleavage activity by heparin or proteinase K digestion in the case of the enzyme cascade model, one has to propose that heparin inhibition of the internal addition of U residues, which is postulated to occur in this model by the TUTase-mediated addition of Us to the 3′-OH, prevents proper religation, thereby causing an increase in the steady state abundance of cleaved molecules. Destruction of the TUTase and the RNA ligase (20) by digestion with proteinase K would also prevent religation from occurring in this model and cause an apparent activation of the cleavage activity.

The micrococcal nuclease digestion evidence for the lack of a requirement for an RNA cofactor for the observed *in vitro* cleavage would appear to contradict the hypothesis of the cascade model that hybridization of pre-edited mRNA with a specific gRNA determines the sites of cleavage. However, the secondary structure of the pre-edited pNB2 CYb mRNA, as shown in the computer-generated "fold" in Fig. 9, emulates the gRNA/mRNA hybrid and presents mismatched bases adjacent to a duplex region. Consistent with this hypothesis is the fact that the 220-nt, 190-nt, and 180-nt minor pNB2 RNA cleavage fragments appear to be derived from cuts at single-stranded regions in this stem-loop structure. We speculate that, *in vivo*, the action of the cleavage enzyme would be limited to the PER in the mRNA/gRNA hybrid and not digest other single-stranded regions due to binding of this factor to an editing complex[3] which undergoes a specific interaction with the PER. This *in vivo* binding has not been examined.

The proteinase K + heparin activation effect can be better explained in terms of the transesterification model for editing (14). It has been shown that, in the case of group I splicing, hydrolysis at normal sites of transesterification occurs in the absence of the guanosine nucleoside which provides the attacking 3′-OH group (25). We propose in the case of our *in vitro* reaction that the observed cleavages of pNB2 RNA represent hydrolysis catalyzed by a protease-resistant catalytic core in the absence of activated gRNA. We propose that the 10–30 kDa "cleavage activity" may actually represent the catalytic core or a portion of the catalytic core of an enzyme involved normally with gRNA/mRNA transesterification. We have shown recently[3] that gRNA in the mitochondrion is not free but is bound to a 13 S TUTase complex of 6–8 proteins. Treatment with heparin produces a decrease in the sedimentation coefficient to 8 S possibly caused by the loss of several proteins from the complex. Proteinase K digestion of the complex releases the bound gRNA. We suggest that only 13 S complex-bound gRNA is active for interacting with the catalytic core enzyme and undergoing 3′-terminal transesterification with mRNA. The heparin treatment or PK digestion of the complex would then lead to hydrolysis at specific mRNA sites catalyzed by the catalytic core. Since hybridization of the mRNA with gRNA is also involved in this model in determining the sites of transesterification *in vivo*, we propose that the observed specificity of hydrolysis sites in the synthetic CYb mRNA in the *in vitro* cleavage experiments is determined by mRNA secondary structure alone and perhaps is limited to single-stranded regions, as discussed above (Fig. 9).

However, micrococcal nuclease digestion of the TL extract

[3] A. Bakker, A. Simpson, and L. Simpson, unpublished results.

in the absence of EGTA does degrade most detectable RNA, but this did not lead to activation of the pNB2 cleavage activity, as would be predicted by the above model. This result could be explained if the bound gRNA active in transesterification is protected from micrococcal nuclease digestion and if this gRNA represents a subset of total gRNA in the extract.

In conclusion, we have shown that a ribonuclease activity can be activated in a kinetoplast-mitochondrial extract by treatment with heparin or by digestion with a protease. We have discussed several possible roles of this activity. More work must be performed to establish the precise role of this activity in mitochondrial RNA processing.

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ABSTRACT: IL-12 is a pluripotent cytokine that interacts with NK and T cells to play a central role in the initiation and maintenance of Th1 responses and IFN-gamma production. Because of the interactive relationship between IL-12 and IFN-gamma response to infectious

IL-12 Enhances Th1-Type Responses in Human *Leishmania donovani* Infections[1]

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IL-12 is a pluripotent cytokine that interacts with NK and T cells to play a central role in the initiation and maintenance of Th1 responses and IFN-γ production. Because of the interactive relationship between IL-12 and IFN-γ response to infectious organisms, a study was undertaken to examine the role of IL-12 in the immune regulation of human visceral leishmaniasis (VL). Human (Hu) VL is associated with immune dysfunction and the appearance of IL-10 mRNA, not present in healed individuals. We found that PBMC from treated VL patients produced both IL-12 p40 and IFN-γ in response to in vitro stimulation with *Leishmania donovani*. The production of both IL-12 p40 and IFN-γ were interdependent and were abrogated by the addition of exogenous Hu rIL-10. In contrast, PBMC from active VL patients did not produce IL-12 p40 or IFN-γ in response to *L. donovani* lysate. Neutralizing anti-IL-10 mAb led to the enhancement of IFN-γ production by active VL PBMC cultured with *L. donovani* lysate, and this enhanced IFN-γ production was blocked by anti-IL-12 mAb. The addition of exogenous Hu rIL-12 to PBMC from active VL patients resulted in the augmentation of IFN-γ in response to *L. donovani* lysate. Therefore, treatment of active VL patient PBMC with anti-IL-10 or IL-12 shifted the response toward a Th1-type response with the production of IFN-γ. These results indicate that IL-12 may play an important role in the regulation of the cellular immune responses in Hu VL. *The Journal of Immunology*, 1995, 154: 4623–4629.

Leishmania are obligate intracellular protozoan parasites of macrophages of their vertebrate hosts. They cause a wide range of infectious processes from asymptomatic to cutaneous, mucosal or visceral manifestations, depending on the species of the parasite and the type of immune response induced. The immune responses and clinical outcome in leishmanial infections are dependent in part on the patterns of cytokines produced (1, 2). Active human (Hu)[4] visceral leishmaniasis (VL) (kala-azar), caused by *Leishmania donovani* and *Leishmania infantum* in Africa, Asia, and Europe or by *Leishmania chagasi* in Latin America, is an acute infection with severe morbidity and high mortality in untreated cases. Patients usually have characteristically high levels of leishmania-specific Abs (3, 4), and do not mount a delayed-type hypersensitivity response to leishmanial Ags (5). In vitro, T cells from VL patients do not proliferate or produce IFN-γ or IL-2 in response to leishmanial Ags (6, 7). We have shown that PBMC from active VL patients produce IL-10 mRNA (8). IL-10 mRNA production was increased in lymphoid tissues taken from patients during active disease versus that observed in post-treatment samples from the same individuals. In contrast, both pre- and post-treatment tissue had readily detectable mRNA for IFN-γ and IL-2 (8). Neutralization of IL-10 with specific mAb led to restoration of both proliferation and IFN-γ production in PBMC from VL patients (8, 9). Similar cytokine patterns were observed in vivo in bone marrow aspirates taken from VL patients before or after treatment (10). These studies demonstrated a role for IL-10 in downregulating T cell immune responses as well as an association between increased IL-10 production and the pathology seen in *L. donovani* infections. In experimental

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[4] Abbreviations used in this paper: Hu, human; VL, visceral leishmaniasis; SAC, *Staphylococcus aureus* Cowan strain.

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systems, it has been shown that the production of IFN-γ is closely associated with healing of *Leishmania major* infections in murine models (11). The augmentation of Th1 and down-regulation of Th2 type responses thus appear to be important for resolution of visceral and cutaneous leishmaniasis. One cytokine known to be capable of these functions is IL-12.

Previously known as NK cell-stimulating factor (12) and cytotoxic lymphocyte maturation factor (13), IL-12 is a heterodimeric cytokine produced by mononuclear macrophages and B-cells. It displays multiple biologic effects on T-cell and NK cell function. The biologic activities of IL-12 include enhancement of NK and T cell proliferation, production of IFN-γ, and cytolytic activity (12, 13). IL-12 plays a key role in the initiation and determination of the type of Th response at the very early stages of antigenic challenge (14–16). As such, it can play an important role in the development of both innate and cognate immune responses to intracellular pathogens. In the murine *Leishmania major* model, IL-12 was shown to induce a Th1-type response and protective immunity (17, 18), and was shown to act as an effective adjuvant for the initiation of protective cell-mediated immunity against murine leishmaniasis (19).

The objectives of this study were to define the role of IL-12 in human *L. donovani* infections, to determine whether IL-12 could augment the cellular immune responses of active VL patients, and to define mechanisms that regulate IL-12 production in *L. donovani* infections.

Materials and Methods

Patient material

Patients with active or treated VL were seen at the kala-azar ward of the University of Khartoum Soba Hospital. Active VL was diagnosed clinically by fever of more than 2 wk duration, hepatosplenomegaly, and pancytopenia. Diagnosis was confirmed by the identification of *Leishmania* amastigotes in impression smears obtained from the lymph node, bone marrow, and/or spleen. Peripheral blood samples were obtained either before or 3 to 6 mo after treatment with sodium stibogluconate (Pentostam; Wellcome Reagent Ltd., London, UK) at 10 mg/kg body weight i.v. Normal donor PBMC were obtained from the American Red Cross, Portland, OR.

PBMC culture and cytokine assays

PBMC were separated by Ficoll-Hypaque (Winthrop Laboratories, New York, NY) and frozen for cellular and cytokine analysis. For proliferation assays, PBMC (3×10^5 cells/well) were cultured in complete medium (RPMI 1640 supplemented with penicillin/streptomycin, 2-ME, L-glutamine, and 10% screened pooled A^+ human serum; Trimar, Hollywood, CA) in 96-well, flat-bottom plates with or without 20 μg protein/ml of total parasite lysate prepared from culture promastigotes of *L. donovani* (World Health Organization designated reference strain MOHM/ET/67/HU3) or 5 μg PHA (Sigma Chemical Co. Immunochemicals, St. Louis, MO) for 5 days. The cells were pulsed with 1 μCi of [^{3}H]TdR (ICN Immunochemicals, Costa Mesa, CA) for the final 18 h of culture and incorporation of label was assessed by liquid scintillation counting. Data are represented as mean cpm of triplicate culture and stimulation index, defined as mean of cpm of cultures with parasite lysate/mean cpm of cultures without lysate. To assess the role of cytokines and anti-cytokines in lymphocyte proliferative responses and cytokine production, PBMC from active or treated VL patients were cultured with Hu rIL-12 (10^6 U/mg), Hu rIL-10 (10^4 U/mg), (gifts from Immunex Corp., Seattle, WA) Hu rIFN-γ (5×10^6 U/mg) (a gift from Genentech, S. San Francisco, CA), anti-IL-12, anti-IL-10, and/or anti-IFN-γ and in the presence or absence of parasite lysate. All reagents were endotoxin free as determined by *Limulus* lysate assay (Immunex Corp., Seattle, WA).

For the cytokine analysis PBMC (2 to 5×10^6/ml) were cultured in RPMI 1640 complete medium, using 0.01% endotoxin-free FCS (HyClone Laboratories, Logan, UT), instead of normal human serum, with recombinant human cytokines or anti-cytokine neutralizing Abs in the presence or absence of *L. donovani* lysate. Supernatants and cells were harvested for IL-12 analysis after 24 h of culture, and for IFN-γ analysis after 72 h. The culture supernatants were analyzed for secreted cytokines as described below.

Cytokine analysis, IFN-γ ELISA

Aliquots of supernatants were assayed for IFN-γ, IL-12, and IL-10. IFN-γ was quantitated by a double sandwich ELISA using mouse anti-Hu IFN-γ mAb (Chemicon, Temucula, CA) and polyclonal rabbit anti-Hu IFN-γ serum. Hu rIFN-γ was used to generate a standard curve.

Cytokine analysis, IL-12 ELISA

IL-12 p40 was measured in cellfree supernatant by RIA as described using the mAb pairs C11.79/C8.6 (20).

Results

Production of IL-12 by PBMC from treated VL patients

IL-12 is associated with multiple biologic functions in the development of immune responses to intracellular infections, and could play an important role in the immunity against *L. donovani* infection. Recovery from VL is associated with the development of in vitro leishmanial Ag-driven PBMC proliferative responses and IFN-γ production. To determine whether IL-12 p40 is involved in the immune regulation of *L. donovani* infection in humans, we examined PBMC from treated VL patients for the production of IL-12 in response to *L. donovani* lysate. In addition, we examined whether the production of IL-12 p40 is regulated by IL-10 and/or IFN-γ. Although the measurement of p40 is an indirect evaluation of bioactive p70, it provides a good indication of IL-12 regulation.

PBMC from treated VL patients and normal controls were cultured with or without *L. donovani* lysate, in the presence or absence of Hu rIL-10, anti-Hu IFN-γ or control mAb for 24 h, and culture supernatants were analyzed for IL-12 p40 by RIA. The IL-12 p40 RIA measures the sum of both the bioactive heterodiameric and inactive homomultimers. PBMC from five treated VL patients produced IL-12 p40 in response to *L. donovani* lysate (Fig. 1). The same lysate preparation did not elicit detectable IL-12 p40 in PBMC from normal controls (Fig. 1). IL-12 p40 production by treated VL PBMC was abrogated by the addition of Hu rIL-10 to the cultures. Similarly, the addition of neutralizing anti-Hu IFN-γ to Ag-stimulated PBMC cultures decreased IL-12 p40 production, suggesting that the production of IL-12 is at least partially dependent on IFN-γ. Thus, PBMC from patients cured of VL responded to leishmanial Ag by producing IL-12 p40, an

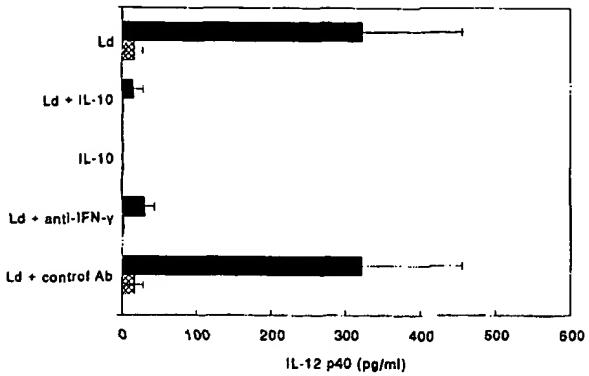

FIGURE 1. IL-12 p40 is produced by PBMC from treated VL patients. PBMC were obtained from individuals 3 to 6 mo after treatment for VL (solid bars) or from normal individuals (hatched bars). Cells were cultured at 1.5 to 2.0 × 10^6/ml in 96-well plates for 24 h with or without 20 μg/ml *L. donovani* lysate (Ld) in the presence or absence of 10 ng/ml Hu rIL-10, 10 μg/ml anti-IFN-γ mAb, or isotype-matched control mAb. IL-12 p40 was determined by RIA in culture supernatants. Data from five patients (mean ± SD) from each group are shown.

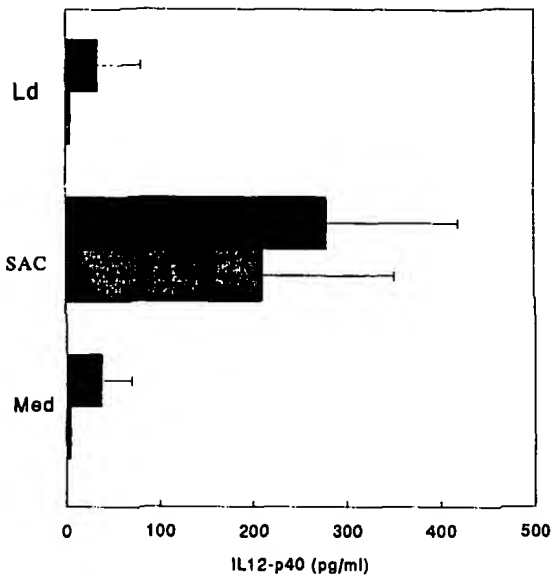

FIGURE 2. PBMC from active VL patients did not produce IL-12 p40 in response to *L. donovani* lysate. PBMC were obtained from five active VL patients (solid bars) or from three normal controls (hatched bars). Cells were cultured at 1.5 to 2.0 × 10^6/ml in 96-well plates for 24 h with or without *L. donovani* lysate (Ld) or SAC. IL-12 p40 was measured by RIA in culture supernatants. Data from five active VL patients and three normal persons(mean ± SD) are shown.

observation consistent with the dominant Th1 type responses seen in these patients (8). In addition, antagonists of Th1 type responses, IL-10 and anti-IFN-γ, blocked the IL-12 p40 by patient PBMC.

Active VL patients PBMC do not produce IL-12 p40 in reponse to *L. donovani* lysate stimulation. Active VL has been associated with the lack of *Leishmania*-driven cellular responses and IFN-γ production. IL-12 is associated with multiple cellular immune responses to infectious organisms. To determine whether the lack of cellular responses seen in active VL is associated with lack in IL-12 production, we examined PBMC from active VL patients for the production of IL-12 in response to *L. donovani* lysate.

PBMC from five active VL patients did not produce IL-12 p40 in response to stimulation with *L. donovani* lysate. The levels were equivalent to background levels (Fig. 2). Normal controls did not produce IL-12 p40 in response to *L. donovani*. Both groups produced IL-12 p40 in response to *Staphylococcus aureus* Cowan strain (SAC).

IL-12-dependent production of IFN-γ by PBMC from treated VL patients

In clinical and experimental leishmaniasis, IFN-γ appears to be the key cytokine involved in macrophage activation, parasite clearance, healing, and protective immunity. The mechanisms that regulate IFN-γ production in VL are not fully understood. Because IL-12 has been shown to play an important role in the production and maintenance of IFN-γ in intracellular infections, we examined whether IL-12 can regulate IFN-γ by PBMC from active VL patients.

PBMC from treated VL patients and normal controls were cultured with or without *L. donovani* lysate, in the presence or absence of either rIL-10 or anti-IL-12 for 72 h, and culture supernatants were analyzed for IFN-γ by ELISA. PBMC from 4 treated VL patients, but not normal individuals, produced high levels of IFN-γ (ranging from 10 to 27.4 ng/ml) in response to *L. donovani* lysate (Fig. 3). The production of IFN-γ was inhibited by IL-10, as expected from our previous study in which we inhibited proliferative responses in these PBMC with IL-10 (8). In addition, neutralizing anti-Hu rIL-12 mAb inhibited leishmania-induced IFN-γ production by PBMC from treated VL patients. These experiments indicate that the *Leishmania*-induced IFN-γ production by treated VL patient PBMC is IL-12 dependent.

Anti-IL-10 enhanced the production of L. donovani-specific IFN-γ by PBMC from VL patients

We have previously shown that PBMC from patients with active VL do not proliferate in response to *L. donovani* Ag but produce IL-10. Furthermore, neutralization of IL-10 augmented their Ag-driven proliferative responses as well as IFN-γ production. This provided evidence that IL-10, a Th2-type associated cytokine with multiple down-regulatory effects, is associated with the lack of *Leishmania*-driven cellular responses in active VL. We examined the possibility that the augmentation of IFN-γ production by

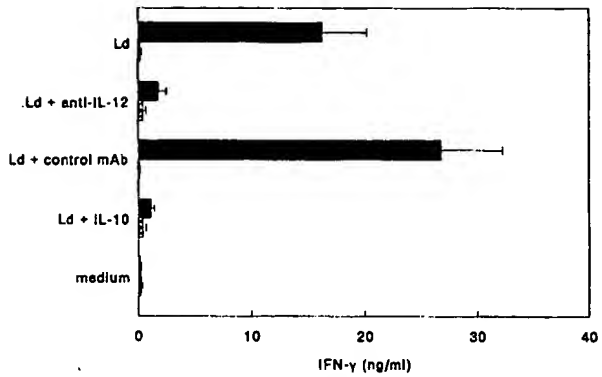

FIGURE 3. IFN-γ is produced by PBMC from treated VL patients. The mean level of supernatant IFN-γ produced by the individuals tested was 19.70 ± 7.00 ng/ml. The production of IFN-γ was inhibited by Hu rIL-10 or by anti-IL-12. PBMC from treated VL patients (solid bars) or from normal individuals (hatched bars) were cultured at 1.5 to 2.0 × 10^6/ml in 96-well plates for 72 h with or without 20 μg/ml of *L. donovani* lysate in the presence or absence of 10 ng/ml Hu rIL-10, 20 μg/ml anti-IL-12 mAb (C8.6), or isotype-matched control mAb. Other cultures received no *L. donovani* lysate (medium). IFN-γ was determined in culture supernatants by ELISA. Data from four patients (mean ± SD) from each group are shown.

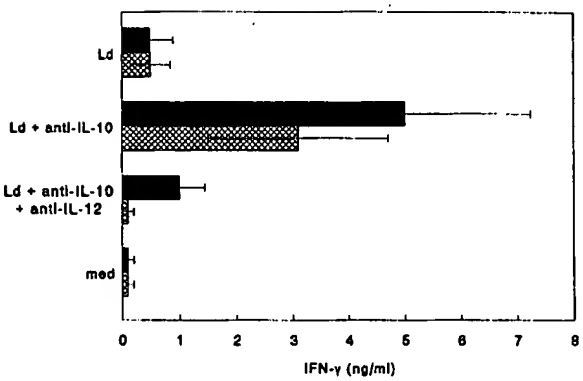

FIGURE 4. Neutralizing anti-IL-10 mAb enhances the production of IFN-γ by PBMC from active VL patients. PBMC from active VL patients (solid bars) or from normal individuals (hatched bars) were cultured at 1.5 to 2.0 × 10^6/ml in 96-well plates for 72 h with or without 20 μg/ml *L. donovani* lysate (Ld) in the presence or absence of 50 μg/ml anti-IL-10 mAb (JES3–19F1), 10 μg/ml anti-IL-12 mAb (C8.6), or isotype-matched control mAb. IFN-γ was determined in culture supernatants by ELISA. Data from five patients (mean ± SD) from each group are shown.

anti-IL-10 in PBMC from acute VL patients is dependent on IL-12.

As previously documented (6), PBMC from acute VL patients did not produce IFN-γ in response to *L. donovani* Ag. Neutralizing anti-IL-10 mAb augmented the production of *L. donovani*-specific IFN-γ in five active VL patients (Fig. 4), as we have reported (9). The addition of neutralizing anti-Hu rIL-12 in combination with anti-IL-10 markedly reduced the production of *L. donovani*-specific IFN-γ. Thus, the neutralization of IL-10 augments *Leishmania*-specific IFN-γ in active VL patients, and this augmented IFN-γ production is IL-12 dependent. Interestingly, the combination of leishmanial Ag + anti-IL-10 in mAb also led to significant IFN-γ production in normal individual PBMC. This observation is consistent with the observed stimulatory effects of leishmanial lysates on human PBMC (21).

IL-12 augmented the production of L. donovani*-specific proliferation and IFN-γ by PBMC from active VL* patients

IL-12 has been shown to be essential for the production and regulation of IFN-γ in intracellular bacterial and parasitic infections. To examine the role of IL-12 as an immunomodulator in VL, PBMC from active VL patients were cultured with or without *L. donovani* lysate, in the presence or absence of rIL-12, and supernatant IFN-γ was assayed after 72 h.

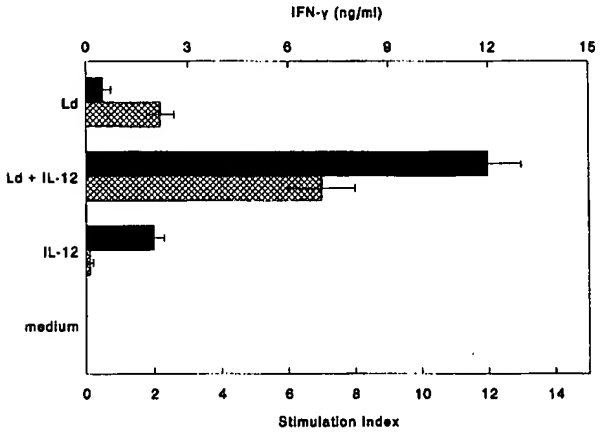

FIGURE 5. IL-12 augmented proliferation (hatched bars) and the production of *L. donovani*-specific IFN-γ (solid bars) by PBMC from patients with active VL. Cells (2 × 10^6/ml) were cultured for 72 h in the presence or absence of 20 μg/ml *L. donovani* lysate with or without 10 ng/ml Hu rIL-12, and the culture supernatants were analyzed for IFN-γ by ELISA. For proliferation data, cultures were incubated as above for five days. Data from 5 patients (mean ± SD) from each group are shown. Stimulation index is represented as cpm of cultures with Ag ÷ cpm of medium control cultures.

PBMC from active VL patients did not proliferate or produce IFN-γ in response to *L. donovani* lysate (Fig. 5). However, the addition of rIL-12 in combination with *L. donovani* lysate to the cultures markedly increased the production of *Leishmania*-driven responses in all individuals tested. IL-12 in the absence of leishmanial lysate did

not increase proliferation or IFN-γ production. The observation that IL-12 can enhance proliferation and the production of IFN-γ in active VL indicate that IL-12 has the potential to restore T cell responses in these patients. The results indicate that IL-12 may enhance Th1-type response in a human infectious disease.

Discussion

Active VL is associated with immune dysregulation and presence of IL-10 whereas healing is associated with a dominant functional Th1-type response (8, 10). In the present study, we found that IFN-γ and IL-12 p40 were produced by PBMC from treated VL patients in response to *L. donovani* lysate. In contrast, cells from active VL patients did not produce IFN-γ or IL-12 p40. The production of these two cytokines after successful treatment was found to be interdependent. This provides evidence that IL-12 and IFN-γ are associated with the healing process and could play an important role in immunity against human VL. The production of both IL-12 and IFN-γ was inhibited by IL-10.

IL-12, a heterodimeric cytokine, has multiple biologic functions on NK and T cells and a central immunoregulatory role on the initiation and maintenance of Th1 responses (12, 13, 15, 16), and is a potent inducer of IFN-γ production (22). There is an intricate relationship between IL-12 and IFN-γ in both innate and cognate immunity against intracellular pathogens (14–16). Production of IFN-γ by Th1 cells appears to be essential for macrophage activation, microbial clearance, healing and protective immunity in leishmanial infections (23). The mechanisms that control the dynamics of interaction between these Th1 related-cytokines and other Th2-related cytokines in human disease are not fully understood. We examined the in vitro cellular responses of PBMC from VL patients during active disease and after treatment to define the role of IL-12 in human *L. donovani* infection.

IL-10, a Th2-type associated cytokine, inhibits Ag-specific cellular responses in active visceral leishmaniasis. It has been shown to induce many inhibitory effects on IFN-γ production and function, on the macrophage capacity of Ag presentation and cytotoxicity (24–27). IL-10 was shown to inhibit human lymphocyte IFN-γ production by suppressing NK cell-stimulating factor/IL-12 synthesis in accessory cells (28). In the present study, the production of IL-12 p40 by PBMC from treated patients was markedly reduced by the addition of anti-IFN-γ. This result agrees with in vitro data demonstrating that IFN-γ can augment IL-12 production (29). The mechanisms underlying the regulation of the Th1/Th2 type responses and macrophage cytokines in human VL are not fully understood. IL-12 plays a central role in the initiation and maintenance of protective Th1 responses and is associated with cure in *L. major* infections (17, 18). However, it is evident from the present study that IL-12 strongly influences Th1 type responses in VL patients, and may be important in the outcome of infection and protective immunity.

PBMC from active VL patients did not produce IFN-γ or IL-12 in response to *L. donovani* lysate. Acute VL has been associated with the lack of *Leishmania*-driven proliferative responses and IFN-γ production (6, 7). In both human and animal infections, the up-regulation of IFN-γ and IL-12 production and function, and the down-regulation of the inhibitory Th2 type responses appear to be critical for parasite clearance and healing. We used two approaches for the reversal of the cytokine pattern associated with the in vitro PBMC responses in active VL. One approach was to neutralize the inhibitory effects of IL-10 on the production of IFN-γ and IL-12 and the other was to augment the production of IFN-γ by the addition of exogenous Th1-type inducing cytokines. We considered IL-12 to be a likely candidate for augmenting the production of IFN-γ PBMC in active VL patients. Indeed, both approaches led to the induction and enhancement of the production of IFN-γ and both were dependent on IL-12. Neutralizing anti-Hu IL-10 mAb led to the induction of IL-12 production by PBMC from two active VL patients in the presence or absence of added *L. donovani* Ag (data not shown). The responses in the absence of added parasite lysate probably reflects the presence of Ag-sensitized cells because they were from an environment in which they were continually exposed to parasite Ag as the result of acute infection.

The results of this study demonstrate that an established dominant Th2-type response may be switched, in vitro, to a Th1-type response using treatment with anti-cytokine (anti-IL-10) or cytokine (IL-12). IL-12 and anti-IL-10 were both very efficient in enhancing the ability of PBMC from active VL patients to produce IFN-γ. In animal models, the down-regulation of Th2 type and upward regulation of Th1-type cytokine responses are critical for parasite clearance and healing. In animal models of leishmanial or nematode infections, the development of a Th2 response has been prevented by IL-12 given before infection (17, 18, 30). Clinically, the immunologic spectrum in acute VL includes a mixed Th1 and Th2 profile, with a dominance of Th2 cytokines (IL-10). The potent ability of neutralizing anti-IL-10 to up-regulate the production of IFN-γ and IL-12 in the presence or absence of *Leishmania* Ag implies significant therapeutic potential. This indicates that anti-IL-10 mAb or IL-10 antagonists could induce in vivo cytokine therapeutic effects and could lead to potentiation of chemotherapy of VL.

In summary, we have shown that IL-12 could augment IFN-γ in active VL PBMC. Similarly, IL-12 was shown to induce Th1-augmenting effects in HIV infections (31). The addition of exogenous Hu rIL-12 augmented the proliferative responses and the production of IL-2 and IFN-γ by PBMC from HIV-infected individuals in response to HIV env Ag and other common Ag. Our observation in active VL, together with finding of IL-12 in the responses

of treated VL patients suggests a possible therapeutic role for IL-12 in VL. It has recently been reported that IL-12 is associated with protective immunity in tuberculosis (32). The unique multiple immnuoregulatory functions of IL-12 make it a likely candidate for cytokine therapy in intracellular infections like leishmaniasis. For example, Hu rIFN-γ and recombinant granulocyte-macrophage CSF cytokines were shown to be effective in potentiating the therapeutic effect of chemotherapy in human VL (33, 34). There is potential for IL-12 to lead to similar in vivo effect in VL and other intracellular infections requiring a functional Th1-type cytokine repertoire. The administration of IL-12 could augment IFN-γ and could induce costimulatory or synergistic effects leading to a switch to a Th1-type response and associated protective immunity. As shown in the murine *L. major* model (19), another role for IL-12 will be as an adjuvant inducing early and stable Th1-type responses in human leishmaniasis.

Acknowledgments

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EXR - NOTES.
Patricia Duffy 09/975,020 1645 1/11
NPL ✓ ADONIS ____ NO ____ Vol No ____ NOS ____
MIC ____ BioTech ✓ Main ____ CK Cite ____ DuplRequest ____
CALL# QR180 .J76 INITIALS WK DATE 1/11

%%%leishmanial%%% antigen that elicits IL-12 production and Th1-type responses in patients as well as IL-12 production in normal human PBMC.

0009719392 BIOSIS NO.: 199598187225
Cytotoxicity in human mucosal and cutaneous %%%leishmaniasis%%%
AUTHOR: Barral-Netto M (Reprint); Barral Aldina; Brodskyn Claudia; Carvalho E M; Reed S G
AUTHOR ADDRESS: Serv. Imunologia-HUPES-UFBA, R. Joao das Botas s/n, 40110-040 Salvador, Bahia, Brazil**Brazil
JOURNAL: Parasite Immunology (Oxford) 17 (1): p21-28 1995 %%%1995%%%
ISSN: 0141-9838
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: CD8+ T cells and lysis of parasitized macrophages seem to be important in the resistance to murine %%%leishmaniasis%%%. In the present study, we evaluated peripheral blood mononuclear cell (PBMC) from patients with either cutaneous (CL) or mucosal (ML) %%%leishmaniasis%%% in cell lysis assays using 51-Cr-labeled Daudi or K562 cells, or autologous antigen-pulsed macrophages as targets. Results are reported as lytic units (number of cells required for 30% lysis) per million PBMC. Exposure of patient PBMC (n = 12) to %%%lysate%%% from %%%Leishmania%%% amazonensis promastigotes led to an increase in cytotoxic activity compared to unstimulated patient cells against Daudi (81.8 +- 14.9 vs 13.6 +- 5 lytic units (LU) per million PBMC; mean +- SEM) and K562 (65.7 +- 8.4 vs 13.1 +- 5 LU/10-6 PBMC). ML had higher responses when CL in

0008772404 BIOSIS NO.: 199395074670
Mapping human T cell epitopes in %%%Leishmania%%% gp63: Identification of cross-reactive and species-specific epitopes
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JOURNAL: Journal of Immunology 150 (3): p932-939 %%%1993%%%
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LANGUAGE: English

0008292116 BIOSIS NO.: 199293135007
A RIBONUCLEASE ACTIVITY IS ACTIVATED BY HEPARIN OR BY DIGESTION WITH PROTEINASE K IN MITOCHONDRIAL EXTRACTS OF %%%LEISHMANIA%%%-TARENTOLAE
AUTHOR: SIMPSON A M (Reprint); BAKALARA N; SIMPSON L
AUTHOR ADDRESS: DEP BIOLOGY MOLECULAR BIOLOGY INSTITUTE, UNIVERSITY CALIFORNIA, LOS ANGELES, CALIF 90024, USA**USA
JOURNAL: Journal of Biological Chemistry 267 (10): p6782-6788 %%%1992%%%
ISSN: 0021-9258
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: A ribonuclease activity in a 100,000 .times. g supernatant of a Triton %%%lysate%%% of a mitochondrial-kinetoplast fraction from %%%Leishmania%%% tarentolae is activated by incubation with heparin or by predigestion of the %%%lysate%%% with proteinase k or pronase. In vitro-transcribed preedited cytochrome b mRNA is cleaved at several

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Mapping Human T Cell Epitopes in Leishmania gp63

Identification of Cross-Reactive and Species-Specific Epitopes[1]

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ABSTRACT. Both a conserved surface metalloprotease of leishmania, gp63 as well as certain gp63-derived peptides, have been shown to have immunoprophylactic potential in mouse models of leishmaniasis. In addition, PBMC from individuals with cutaneous, mucosal, or cured visceral leishmaniasis respond in vitro to both native and rgp63. In this report, we mapped human T cell epitopes within gp63. T cells from leishmaniasis patients responded in vitro to certain peptides of gp63 by proliferation and IFN-γ production. One peptide, (PT7), stimulated cells from all individuals tested (n = 7). Anti-PT7 T cell lines derived from PBMC of a mucosal leishmaniasis patient contained a heterogeneous population of cells which responded by proliferation and IFN-γ production to in vitro stimulation with *Leishmania* promastigote lysate. Another peptide (PT1) derived from *Leishmania chagasi* gp63 stimulated PBMC from an *L. chagasi* patient although the corresponding *Leishmania major*-derived peptide did not. Both *L. major* PT7 and *L. chagasi* PT1 were able to induce anti-*Leishmania*-specific T cell lines from normal human PBMC. These T cell lines responded to in vitro stimulation with promastigote lysate indicating that both peptides were immunogenic for naive T cells in vitro. In conclusion, both antigenic and immunogenic gp63 peptide sequences have been defined, some appearing to be conserved among *Leishmania* species and at least one that appears to be species specific. *Journal of Immunology,* 1993, 150: 932.

Leishmania are parasitic protozoa transmitted to mammals by the bite of an infected sandfly. The parasite exists both as an infective promastigote and an obligate intracellular amastigote that replicates within macrophages. Human infections are generally manifested as localized cutaneous lesions, mucosal infections of varying severity, or subclinical to acute visceral disease. Despite the range of clinical disease, recovery from all forms of leishmaniases appears to be dependent on specific T lymphocyte responses (1). Products of activated T cells including IL-2 (1), IFN-γ (2–5), and granulocyte-macrophage CSF (6) have been demonstrated to be important mediators of disease resolution and are being tested in the clinical treatment of cases refractory to standard antimonial therapy (7, 8).

The identification of defined parasite proteins and peptides that induce or elicit beneficial T cell responses may contribute to vaccine development. One widely studied parasite Ag is a 63-kDa surface glycoprotein (gp63), highly conserved amongst *Leishmania* species (9, 10).

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Both native gp63 and rgp63 expressed in *Salmonella* have been reported to partially protect mice from infection with *Leishmania* (11, 12). More recently, murine T cell epitopes within gp63 have been identified and shown to induce partial protection against *L. major* infection (13).

Recently, we reported that gp63 was a potent stimulator of human T cell responses. T cells from patients recovered from leishmaniasis proliferated and produced IFN-γ when stimulated with gp63, and in vitro immunization with native gp63-induced T cells from normal individuals to proliferate and secrete IFN-γ in response to *Leishmania* lysate (14). In the present report, we mapped human T cell epitopes within *Leishmania chagasi* and *Leishmania major* gp63 and demonstrated that several T cell epitopes stimulated both proliferation and IFN-γ production in recovered leishmaniasis patients. In addition, we found species variability in one T cell epitope of gp63, with an *L. chagasi* patient responding to the *L. chagasi* peptide, but not the *L. major* peptide.

Materials and Methods

PBMC

Peripheral blood was obtained from individuals living in areas of Brazil endemic for leishmaniasis who had clinical disease or a recent history of disease confirmed by both parasitologic and serologic evaluation, as described (15, 16). These included patients with active or recently treated (<2 yr) cutaneous leishmaniasis (*L. amazonensis* infection), active mucosal leishmaniasis (*L. braziliensis* infection) or patients with visceral leishmaniasis (*L. chagasi* infection) at 3 to 6 mo after standard antimonial chemotherapy. Cutaneous disease patients were selected on the basis of having active lesions, positive DTH responses (>5 mm in duration) and in vitro proliferative responses (stimulation index >10) to promastigote lysate. It is our experience that PBMC which have relatively low responses to *Leishmania* lysate do not respond well in vitro to gp63 or its derived peptides. PBMC were isolated from whole blood by density centrifugation through Ficoll-Paque (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Leishmania Ag

Soluble lysate was prepared by freeze/thaw lysis of *Leishmania* promastigotes obtained from stationary phase cultures as described previously (17). rgp63 from *L. major* was produced in *Escherichia coli* using a T7 RNA polymerase expression system and the pET plasmid vectors (18). rgp63 was isolated from an insoluble inclusion body fraction in the presence of 8 M urea by preparative isoelectric focusing followed by ammonium sulfate fractionation as previously reported (14, 19). Peptides from *L. major* were synthesized using Merrifield solid phase methodologies as described previously (13). Peptides from *L. chagasi* were synthesized via Merrifield methodology using Fmoc chemistry on an Applied Biosytems 430A peptide synthesizer (Applied Biosystems, Foster City, CA. 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate was used to generate the activated amino acids that were protected as follows: Thr, Ser, Asp, Glu, and Tyr as t-butyl; Asn, Gln, Cys, and His as Trtyl; Lys as t-Boc and Arg as Pmc. Peptides were cleaved from their solid support using trifluoroacetic acid, in the presence of phenol, thioanisole, and ethanedithiol, for a period of 2 h at 25°C, before being precipitated into methyl t-butyl ether. The precipitated peptide was washed twice with diethyl ether, air-dried, and purified on a Vydac C18 reverse phase column (2.2 × 25 cm). Pure fractions were combined before lyophilization. Amino acid analysis on a Beckman 6300 amino acid analyzer (Becknam Instruments, Fullerton, CA) was used to determine composition and quantity of each peptide and molecular weights were verified by plasma desorption mass spectrometry on a Bio-Ion 20 mass spectrometer (Applied Biosystems).

T cell lines

T cell lines were generated by culturing PBMC with *Leishmania* Ag (native gp63, 5 μg/ml; PT7, 50 μg/ml; or PT4 50 μg/ml) in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with gentamycin, 2-ME, L-glutamine, and 10% screened pooled human AB serum (Trimar, Hollywood, CA). After 6 days in culture, human rIL-2 (10 ng/ml) was added to expand Ag reactive T cells. Thereafter, medium was replaced every 3 to 4 days with fresh medium containing rIL-2. At 12–16 day intervals, the cells received Ag and irradiated (3500 rad) autologous PBMC.

In vitro proliferation assays.

PBMC from normal individuals or from patients with either cutaneous or mucosal leishmaniasis were cultured at 4 × 10^5 cells/well in 96-well flat-bottom plates in complete medium (200 μl/well) in the presence or absence of Ag for 5 days. The wells were pulsed with 1 μCi of [^{3}H]TdR (ICN Immunochemicals, Costa Mesa, CA) for the final 18 h of culture. In some instances, cell culture supernatants (75 μl/well) were harvested for cytokine assays prior to adding [^{3}H]TdR. Initially, peptides were tested at concentrations ranging from 10 to 100 μg/ml. Our studies revealed that optimal proliferative responses were obtained with 50 μg/ml.

To assess the Ag reactivity of T cells in continuous culture, T cell lines were tested in a modified proliferation assay. Rested T cells were cultured in 96-well round-bottom plates at 2 × 10^4 cells/well with 1 × 10^5 irradiated (3500 rad) autologous or 2 × 10^5 matched heterologous PBMC with or without Ag. Cell culture supernatants were

harvested 48 or 72 h after Ag stimulation. On the third day of culture, the wells were pulsed with 1 μCi of [^{3}H]TdR for 6 h. Data are represented as mean cpm × 10^{-3} ± SD. The stimulation index was calculated as mean cpm of cells cultured with Ag/mean cpm of cells cultured without Ag.

Cytokine assays

IFN-γ was quantitated in cell culture supernatants by RIA (Centocor, Malvern, PA), or by a double sandwich ELISA using a mouse anti-human IFN-γ mAb (Chemicon, Temucula, CA) and a polyclonal rabbit anti-human IFN-γ. Human rIFN-γ (Genentech Inc., South San Francisco, CA) was used to generate a standard curve.

Immunofluorescence and flow cytometry

Cells from Ag specific lines were tested for CD8 and CD4 surface marker expression. T cells were preincubated with 1% normal rabbit serum in PBS/0.02% NaN_3 for 30 min at 4°C to prevent nonspecific FcR binding of test antibody. Cells were washed once in PBS/NaN_3 and stained with phycoerythrin-conjugated Leu 2a (anti-CD8) and FITC-conjugated Leu 3a (anti-CD4) (Becton Dickinson Immunocytometry Systems, Mountain View, CA) for 30 min at 4°C. The cells were washed 3x in PBS/NaN_3, fixed in 0.1% paraformaldehyde and analyzed by flow cytometry using a single laser FACScan (Becton Dickinson). A minimun of 5000 cells was analyzed per sample.

In vitro sensitization of normal T cells with peptides of gp63

T cells from normal individuals were sensitized in vitro with peptides of gp63 as described previously with some modifications (14). Briefly, PBMC (1–2 × 10^7 cells/ml) from normal individuals were cultured with 50 to 100 μg/ml of peptide and 1000 U human IFN-γ (Genentech Inc.). After 5 to 7 days in culture, T cell blasts were enriched by centrifugation through Ficoll-Hypaque and recultured with irradiated APC (individual autologous PBMC), peptide, and IL-2. Thereafter, these T cell lines were propagated in a manner similiar to that described for T cell lines generated from leishmaniasis patients.

Results

Characterization of patient PBMC responses to peptides of *L. major* gp63

Thirteen peptides were synthesized corresponding to amino acid sequences of *L. major* gp63 which were predicted to contain T cell epitopes by an algorithm based on amino acid motif (20) (Table I). Murine responses to seven of these peptides (PT1–4 and PT6–8) have been reported previously (13). Peptides were tested for their ability to stimulate PBMC from patients with cutaneous, mucosal, or cured visceral leishmaniasis. Several peptides consistently stimulated patients' PBMC, including PT4, PT7, and PT8 (Fig. 1). Other peptides also elicited proliferative responses (PT9, PT13, and PT15) but these occurred in fewer patients and were generally of a lower magnitude. When tested on a gp63-specific T cell line generated from a patient with cutaneous leishmaniasis, it was again observed that PT4, PT7, and PT8 elicited the strongest proliferative responses (Fig. 1 *4*). These results demonstrate the ability of peptides from conserved regions of *L. major* gp63 to elicit responses from patients infected with heterologous *Leishmania* species.

IFN-γ production was tested in supernatants harvested from stimulated PBMC of four leishmaniasis patients. Stimulation with either PT4, PT7, or PT8 elicited the secretion of IFN-γ from all individual patient PBMC tested (Table II). PT15 elicited IFN-γ production in one of three patients tested. These results indicate that certain peptides of gp63 induce strong proliferative responses as well as the production of IFN-γ in individuals with a different clinical forms of leishmaniasis.

Table I
Synthesized peptides of L. major and L. chagasi gp63

Peptide[a]	Position[b]	Sequence[c]
1	1–14	VRDVNWGALRIAVS ••AA••••••••••
2	48–62	LTNEKDDILVKHLIP •••••••••••••••
9	61–79	IPQAVQLHTERLKVQQVQG
10	116–130	VPSEEGVLAWATTCQ •••••••••••••••
3	154–169	YDQLVTRVVTHEMAHA ••••••••••••••••
4	159–174	TRVVTHEMAHALGFSVG ••••••••••••••••
11	171–187	GFSGPFFEDARIVANVP
12	199–214	INSSTAVAKAREQYGC ••••••••••••••••
13	212–227	YGCDTLEYLEVEDQGG
15	262–277	FGDLGFYQADFSKAEV
6	379–394	PFNVFSDAARCIDGAF
7	385–401	AARCIDGAFRPKATDG ••••••••••TSH•
8	395–410	RPKATDGIVKSYAGLC •••TSH••••••••••

[a] Peptide designation.
[b] Position of peptide within mature gp63 sequence.
[c] *L. major* gp63 peptide sequence shown on top. Underneath, identical *L. chagasi* residues are indicated by a dot and differences denoted by the single letter amino acid code.

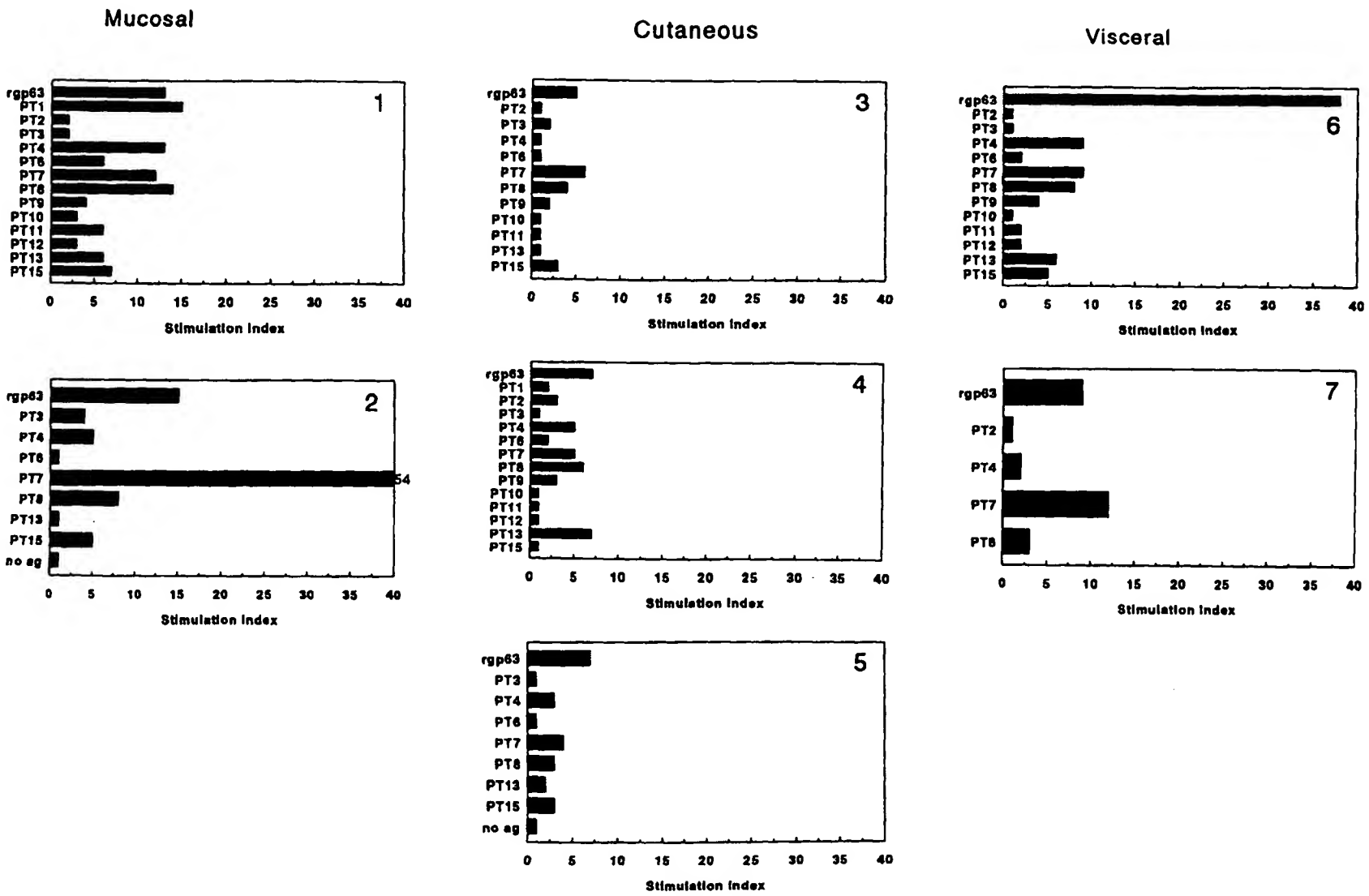

FIGURE 1. PBMC from mucosal (*1* and *2*), cutaneous (*3* and *5*) and visceral (*6* and *7*) leishmaniasis patients were tested with *L. major* rgp63 (20 μg/ml) and peptides of gp63. *4* is a T cell line generated from a cutaneous patient's PBMC against *L. amazonensis* native gp63. Cells were also stimulated with promastigote lysate: *L. brasiliensis* (mucosal, *1*, stimulation index = 57; *2*, stimulation index = 140); *L. amazonensis* (cutaneous; *3*, stimulation index = 21; *4*, stimulation index = 6; *5*, ND) and *L. chagasi* (visceral, *6*, stimulation index= 100; *7*, stimulation index = 146).

Table II
IFN-γ production by PBMC in response to peptides of L. major gp63

Ag	IFN-γ (U/ml)		
	JS[a]	SR	AB
Lmrgp63	56[b]	56	22
PT3	0	0	0
PT4	17	4	4
PT6	0	0	0
PT7	64	4	15
PT8	19	8	8
PT13	0	0	NT
PT15	1	0	0
No Ag	0	0	0

[a] PBMC from leishmaniasis patients were incubated with *L. major* rgp63, 20 μg/ml or peptides of gp63, 50 μg/ml. Supernatants (100 μl/well) were harvested on day 5.
[b] Data are expressed as NIH reference units for IFN-γ.

Characterization of peptide-specific T cell line

To characterize the cells responding to peptides of gp63, a T cell line against PT7 was generated from PBMC of a cutaneous leishmaniasis patient (CC). This peptide was chosen because it elicted strong responses in most of the patients tested. The T cell line generated against PT7 proliferated strongly and produced very high levels of IFN-γ in response to promastigote lysate as well as to PT7 (Table III). The T cells did not respond to rgp63. This is not surprising as we have observed some differences in T epitope selection or dominance between the native and recombinant forms of gp63 (14). This T cell line was specific for PT7 as it was not stimulated by other peptides of gp63 (not shown). The FACS profile of two anti-PT7 specific T cell lines demonstrated the presence of $CD4^+$ and $CD8^+$ positive T cells. This is of particular interest because it suggested that a single epitope could induce more than one population of T cells (Fig. 2).

Identification and fine specificity mapping of *L. chagasi* species-specific T cell epitope

Although several *L. major* gp63 peptides elicited T cell responses in patients infected with other species of *Leishmania*, we wished to determine whether responsiveness could be enhanced using peptides corresponding to the gp63 sequence of the infecting species. To test this, peptides of *L. chagasi* gp63 were synthesized that corresponded to selected regions of the *L. major* sequence. These included peptides that had been shown to elicit responses in leishmaniasis patients as well as those that did not (Table I). These peptides were then tested on PBMC of an individual recently cured of visceral leishmaniasis caused by *L. chagasi* infection. *L. chagasi* peptides, PT2, 4, 7, and 8 elicited the same responses (not shown) as their *L. major* counterparts despite varying degrees of sequence differences (Table I) again demonstrating conservation of

Table III
Peptide specific T cell lines generated from a patient with cured mucosal leishmaniasis

Ag[a]	C. PT7[b]	
	x̄ cpm (SD)	IFN (U/ml)
Lysate	1555 (588)	70
rgp63	538 (81)	0
PT7	1588 (340)	40
PT8	377 (9)	0
No Ag	335 (110)	0

[a] Ag were used at the following concentrations: *L. major* lysate, 20 μg/ml; *L. major* rgp63, 20 μg/ml; gp63-peptides, 50 μg/ml.
[b] Peptide specific T cell line.

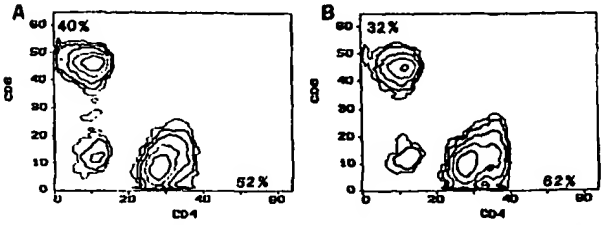

FIGURE 2. FACS profiles of anti-PT7-specific T cell lines generated from PBMC of two patients CC (*A*) and AB (*B*). T cells were double stained with PE-conjugated anti-CD8 and FITC-conjugated anti-CD4 and analyzed with FACScan. The percentage of $CD4^+$ and $CD8^+$ T cells is shown.

T cell epitopes of gp63. In contrast, the two PT1 peptides elicited different degrees of proliferation (Table IV). *L. chagasi* PT1 stimulated proliferation of T cells from SR, although the corresponding *L. major* peptide did not. The sequences of these two peptides are shown in Table I. There are two amino acid differences between *L. chagasi* and *L. major* PT1. To determine the residue(s) critical to the activity of the *L. chagasi* peptide, two variant peptides were synthesized with substitutions at one of these sites (Table IV). Variant peptide Lc 1.1 was synthesized with a D residue substituted for A at position 3 and variant peptide Lc 1.2 was synthesized with a V residue substituted for A at position 4. Neither variant peptide stimulated PBMC of SR as well as Lc PT1 (Table IV).

In vitro immunization of normal PBMC with peptides of gp63

The ability of several peptides of gp63 to induce appropriate T cell responses in naive cells was assessed. T cell lines were generated using PBMC of normal uninfected individuals by in vitro immunization with peptides PT7 and PT3 of *L. major*, and PT1 of *L. chagasi*. T cells from these lines were tested for the ability to respond to challenge with *Leishmania* lysate, rgp63, or the inducing peptide. Four anti-PT7 and three anti-PT1 T cell lines generated from four different donors proliferated in response to promastigote lysate or rgp63 (Fig. 3). Most of the lines proliferated to the inducing peptide, although some did

Table IV
Proliferation of PBMC from cured visceral leishmaniasis patient[a] to Gp63 peptides of L. major and L. chagasi

Ag	μg/ml	cpm (×10⁻³) ± (SD)	S.I.
Lc lysate	10	26,145 (2,945)	54
Lc rgp63	20	5,653 (809)	12
Lm rgp63	20	3,537 (391)	7
Lm PT1	100	374 (195)	1
	25	560 (134)	1
Lc PT1	100	3,071 (1,089)	6
	25	3,966 (974)	8
Lc PT1.1	100	382 (167)	1
	50	1,920 (48)	4
	25	1,010 (246)	2
Lc PT1.2	100	425 (180)	1
	50	1,587 (239)	3
	25	681 (420)	1
Medium		478 (102)	
Lc PT1.1		V R D A N W G A L R I A V S	
Lc PT1.2		V R A V N W G A L R I A V S	

[a] PBMC from patient (SR) with clinically cured visceral leishmaniasis from *L. chagasi* infection. The asterisk denotes substitution in amino acid residue at the designated position.

not. This is probably due to differences in APC uptake and processing of peptide vs protein. In contrast, lines generated with PT3 did not respond well to any of the *Leishmania* Ag tested. Attempts to generate more responsive lines against PT3 with additional donors were unsuccessful (not shown). Thus it was observed that at least one peptide of *L. major* gp63 (PT7) and one peptide of *L. chagasi* gp63 (PT1) can prime cells to respond to promastigote lysate.

Discussion

An important aspect of characterizing T cell epitopes from microbial proteins is defining the nature of the T cell responses induced or elicited. Previously, we analyzed human T cell responses to gp63, which elicited vigorous proliferation and IFN-γ production from patient PBMC and induced T cell responses in normal uninfected PBMC. Although regions of *L. major* gp63 that stimulate murine T cell responses have been described (13, 21), it was not known which regions would stimulate human T cells. We report the mapping of human T cell epitopes of gp63 and the characterization of T cell responses to specific peptides.

Initially, 13 peptides derived from *L. major* gp63 were tested in PBMC from cutaneous, mucosal, and cured visceral leishmaniasis patients that were infected with one of the American species of *Leishmania*, which does not include *L. major*. All of the patients' cells responded to PT7 and most responded to PT4 derived from the *L. major* gp63 sequence. This is not surprising as gp63 is highly conserved among the species of *Leishmania* and we (14)

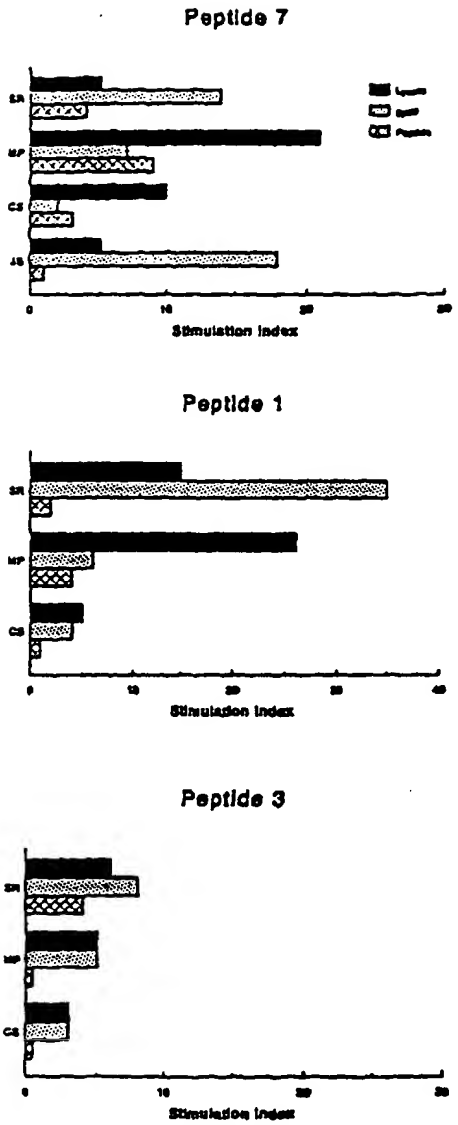

FIGURE 3. T cell lines generated with PBMC from normal uninfected individuals against *L. major* peptides PT7 and PT3 and *L. chagasi* PT1 were tested in a proliferation assay. Cells from each T cell line were stimulated with *L. major* or *L. chagasi* lysate, 10 μg/ml; rgp63, 20 μg/ml and the peptide with which the line was induced, 50 μg/ml. The data are represented as stimulation indices.

and others (18) have previously reported cross-reactive T cell responses to the complete protein, although cross-reacting epitopes had not been previously defined. The identification of conserved T cell epitopes in gp63 is of particular importance in light of the variability of T cell epitopes in major Ag of other protozoa, most notably the circumsporozoite protein of *Plasmodium* (22). This variability at the sites of dominant T cell epitopes may be one mechanism of parasite survival. We have thus identified conserved T cell epitopes of a major Ag of *Leishmania*.

Although the finding that cross-reactive epitopes existed in gp63 is significant, it was possible that dominant species-specific epitopes also existed. Therefore, we compared the abilities of homologous peptides synthesized from *L. major* and *L. chagasi* gp63 to elicit T cell responses in PBMC from an *L. chagasi* patient. Of five peptides tested in this manner one *L. chagasi* peptide (PT1) stimulated this patient's PBMC although the *L. major* counterpart did not. Fine specificity mapping of this sequence demonstrated that the epitope reactivity was localized to two residues, both of which appeared important in maintaining activity of the peptide. Because cells from only one *L. chagasi* patient were tested in this manner, the influence of MHC genes was not explored. However, a more extensive study of peptides of *L. chagasi* gp63 is in progress that will address MHC restriction as well as identify other species-specific and cross-reactive peptides.

PBMC from all of the patients tested proliferated and secreted IFN-γ in response to stimulation with PT4 and PT7. In addition, a PT7-specific T cell line generated from a leishmaniasis patient responded to *Leishmania* lysate with IFN-γ production. These data suggest that these two T cell epitopes can induce a Th1 type of response during natural infection with *Leishmania* parasites. Both murine (23, 24) and human studies (1) strongly suggest that recovery from leishmaniasis depends on the preferential stimulation of a Th1 immune response, with the production of IFN-γ being of critical importance. Both PT4 and PT7 appear to encode major human Th1-stimulating epitopes. The identification of such epitopes is an important step in the evaluation of these potential immunogens.

To characterize the patient cells responding to these peptides, a T cell line against PT7 was generated using PBMC from a patient. This T cell line contained both $CD4^+$ and $CD8^+$ cells and responded to in vitro challenge with lysate by proliferation and IFN-γ production. Similarly, a PT4-specific T cell line generated from this patient also contained both cell types. While the importance of $CD8^+$ T cells in human leishmaniasis is not defined, it is of interest that these peptides can stimulate both cell types. Further work will determine whether these particular peptides actually contain more than one T cell epitope or if in fact one epitope is responsible for stimulating both $CD4^+$ and $CD8^+$ T cells.

We extended our observations on the antigenicity of gp63 peptides, to a preliminary evaluation of the immunogenicity of these peptides in normal human PBMC. Using an vitro immunization system, T cell lines were generated against *L. major* PT7 and PT3 and *L. chagasi* PT1. We found that both PT7- and PT1-sensitized cells responded to in vitro challenge with either *Leishmania* lysate or gp63. In contrast, most of the PT3-specific T cell lines were not responsive to these Ag. Thus, in humans PT3 appears to be neither antigenic nor highly immunogenic, even though this peptide has previously been reported to contain a murine T cell epitope (13) that has been shown to be partially protective in a murine model of leishmaniasis. In contrast, PT4 which overlaps PT3 by 11 residues, elicited responses in five of seven patients tested and induced *Leishmania*-specific responses in normal T cells. These data emphasize the importance of preliminary testing of T cell epitopes in human systems as the dichotomy between murine and human T cell epitope usage is becoming clearer. The described in vitro sensitization system may provide one method with which to obtain a preliminary evaluation of the immunogenic potential of these peptides in humans.

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EXR NOTES.

Patricia Duffy
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visceral %%%leishmaniasis%%%
AUTHOR: Badaro R (Reprint); Benson D; Eulalio M C; Freire M; Cunha S; Netto E M; Pedral-Sampaio D; Madureira C; Burns J M; Houghton R L; David J R; Reed S G
AUTHOR ADDRESS: Infect. Dis. Res. Unit, Hosp. Univ. Prof. Edgard Santos, Univ. Federal Bahia, Rua Joao Botas, s/n Canela, 40110-160 Salvador, Bahia, Brazil**Brazil
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ISSN: 0022-1899
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RECORD TYPE: Abstract
LANGUAGE: English

0010049300 BIOSIS NO.: 199598517133
Human T-cell activation by 14- and 18-kilodalton nuclear proteins of %%%Leishmania%%% infantum
AUTHOR: Suffia Isabelle; Quaranta Jean-Francois; Eulalio Maria C M; Ferrua Bernard; Marty Pierre; Le Fichoux Yves; Kubar Joanna (Reprint)
AUTHOR ADDRESS: Groupe Recherche Immunopathologie Leishmaniose, Lab. Parasitologie, Faculte Med., Ave. Valombrose, 06107 Nice Cedex 02, France **France
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Characterization of a %%%Leishmania%%% tropica antigen that detects immune responses in Desert Storm viscerotropic %%%leishmaniasis%%% patients
AUTHOR: Dillon Davin C; Day Craig H; Whittle Jacqueline A; Magill Alan J; Reed Steven G (Reprint)
AUTHOR ADDRESS: Infectious Disease Res. Inst., Seattle, WA 98104, USA**USA
JOURNAL: Proceedings of the National Academy of Sciences of the United States of America 92 (17): p7981-7985 1995 %%%1995%%%
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RECORD TYPE: Abstract
LANGUAGE: English

0009845824 BIOSIS NO.: 199598313657
IL-12 enhances Th1-type responses in human %%%Leishmania%%% donovani infections
AUTHOR: Ghalib Hashim W; Whittle Jacqueline A; Kubin Marek; Hashim Faisal A ; El-Hassan Ahmed M; Grabstein Kenneth H; Trinchieri Giorgio; Reed Steven G (Reprint)
AUTHOR ADDRESS: Infect. Dis. Res. Inst., 1124 Columbia St., Suite 464, Seattle, WA 98104, USA**USA
JOURNAL: Journal of Immunology 154 (9): p4623-4629 1995 %%%1995%%%
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ABSTRACT: IL-12 is a pluripotent cytokine that interacts with NK and T cells to play a central role in the initiation and maintenance of Th1 responses and IFN-gamma production. Because of the interactive relationship between IL-12 and IFN-gamma response to infectious

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kala-azar in an endemic area of Brazil (Sao Goncalo do Amaranto, RN).
Silva, V. O. da; Borja-Cabrera, G. P.; Correia Pontes, N. N.; Souza, E. P. de; Luz, K. G.; Palatnik, M.; Sousa, C. B. P. de
Instituto de Microbiologia, 'Prof. Paulo de Goes', Cidade Universitaria, CCS, Universidade Federal do Rio de Janeiro (UFRJ), CP 68040, Ilha do Fundao, CEP 21941-590, Rio de Janeiro, Brazil.
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Language: English Record Type: Abstract
Document Type: Journal article

Protection against canine kala-azar was investigated in naturally exposed dogs of an endemic area in Sao Goncalo do Amaranto, Rio Grande do Norte, Brazil, vaccinated with the fucose mannose ligand (FML)-vaccine of %%%Leishmania%%% donovani . The dog vaccination trial stated in December 1996. A total of 97% of vaccinees were seropositive to FML and 100% showed intradermal reaction to L. donovani %%%lysate%%% , 7 months after vaccination. The absorbency values and size of intradermal reaction were both significantly higher in vaccinees than in controls (ANOVA, P <0.0001). After 2 years, 92% (chi SUP 2 =6.996; P < 0.0025) protection was achieved: only 8% of vaccinees showed mild signs of kala-azar with no deaths while 33% of controls developed clinical or fatal disease. The number of human cases in the area decreased from 15 cases in 1996 to 6 cases in July 1997 and to zero in May 1998. The FML-vaccine induced a significant, long-lasting and strong protective effect against canine kala-azar in the field.

0010049300 BIOSIS NO.: 199598517133
Human T-cell activation by 14- and 18-kilodalton nuclear proteins of Leishmania infantum
AUTHOR: Suffia Isabelle; Quaranta Jean-Francois; Eulalio Maria C M; Ferrua Bernard; Marty Pierre; Le Fichoux Yves; Kubar Joanna (Reprint)
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JOURNAL: Infection and Immunity 63 (10): p3765-3771 1995 1995
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ABSTRACT: Leishmanial antigens which stimulate T lymphocytes from primed individuals may be candidates for a vaccine. We recently found a significant concordance between the humoral response specific for two proteins from Leishmania infantum promastigotes, p14 and p18, and a positive leishmanin delayed-type hypersensitivity reaction, testifying to the occurrence of cell-mediated immunity. In t

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Human T-Cell Activation by 14- and 18-Kilodalton Nuclear Proteins of *Leishmania infantum*

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Leishmanial antigens which stimulate T lymphocytes from primed individuals may be candidates for a vaccine. We recently found a significant concordance between the humoral response specific for two proteins from *Leishmania infantum* promastigotes, p14 and p18, and a positive leishmanin delayed-type hypersensitivity reaction, testifying to the occurrence of cell-mediated immunity. In this communication, we describe a partial characterization of these antigens and an in vitro analysis of their capacity to activate primed human T cells. We showed, by immunofluorescent staining and through analysis of subcellular fractions by Western immunoblotting, that in stationary-phase promastigotes, p14 and p18 were located only in the parasite nuclei; in the middle of the log phase, a transitory and only weak expression outside the nucleus was detected. We then showed that p14 and p18 antigens shared a common epitope(s). Finally, we analyzed the in vitro proliferation and interleukin-2 production induced by leishmanial proteins in human peripheral blood mononuclear cells from sensitized subjects. We showed that in some individuals who have been exposed to *L. infantum* the specific response to the whole lysate was mostly due to the nuclear antigens. We demonstrated directly the capacity of nitrocellulose-bound p14 and p18 to activate in vitro all of the tested primed peripheral blood mononuclear cells, which contrasted with a lack of stimulatory activity of other membrane-bound leishmanial proteins. Taken together, our results suggest that an antigenic determinant(s) dominant for some individuals might exist on both antigens.

Infection with parasitic protozoa of the genus *Leishmania* affects mammalian hosts, particularly humans, in the Old World and the New World. It causes a spectrum of disease, depending on the host and on the *Leishmania* species involved. In humans, the infection ranges from self-healing lesions to disseminated cutaneous disease or highly destructive mucosal lesions and from asymptomatic infection to fatal visceral dissemination, causing one of the world's major health problems. The development of vaccines is the essential aim of studies on leishmaniasis. Moreover, the research on immunopathology of parasitic infections has led to highly pertinent models for understanding several aspects of regulation in the immune system (25, 35). Infection with *Leishmania major*, an agent of human cutaneous leishmaniasis, is at present the best-documented example of the differential activation of the Th1 $CD4^+$ lymphocyte subset in resistant strains of inbred mice and the Th2 subset in susceptible strains (6, 7, 24, 33). In this model, the production of Th1-type cytokines (interleukin-2 [IL-2] and gamma interferon) and that of Th2-type cytokines (IL-4, IL-5, and IL-10) are mutually exclusive. However, the situation appears less well defined, even in the murine system, for infection with *L. donovani*, which is responsible for visceral disease. Indeed, the functional studies with inbred strains have failed to correlate the long-term outcome of the disease with differential expansion of the T-cell subsets (12). Recent data obtained in studies with humans demonstrate that Th1- and Th2-like responses coexist in patients cured of visceral leishmaniasis (11, 13–15). At present, there are no data indicating that an association between the expansion of one of the two subsets could lead either to visceral disease or to establishment of asymptomatic infection.

Among several factors, not all of which are known, determining the balance between T-cell subsets and the outcome of infection are the characteristics of leishmanial antigens. It was recently reported that some T-cell epitopes that are protective in the murine host did not elicit immune response in humans (28); this result emphasizes the importance of testing leishmanial antigens with human cells. In this work, we examine two *L. infantum* proteins of 14 kDa (p14) and 18 kDa (p18). A potential use of these antigens in the diagnosis and epidemiology of human visceral leishmaniasis was recently described (19): a Western immunoblot analysis of antibodies to *L. infantum* in patients with visceral leishmaniasis showed that p14 and/or p18 was recognized by virtually all tested patient sera. Recently, in an epidemiological survey carried out in a region in Southern France where *Leishmania* infection is endemic, we found that over 80% of asymptomatic individuals presenting positive leishmanin skin reaction had detectable antibodies against p14 and/or p18 (17). The leishmanin skin test is an indicator of delayed-type hypersensitivity (DTH); it is performed with a suspension of phenol-killed parasites, i.e., with total leishmanial antigens. We thus investigated whether this biological concordance between results of the skin test and of the Western blot (17) was merely fortuitous or whether p14 and/or p18 might contribute not only to humoral but also to cell-mediated specific immunity. First, we examined some of their characteristics in *L. infantum* promastigotes. Then we analyzed their potential to stimulate in vitro human T cells primed in vivo.

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MATERIALS AND METHODS

Parasites, media, and culture. *L. infantum* MON1 (MHOM/FR/81/LPN5) promastigotes were grown at 25°C in RPMI 1640 medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum. This will be referred to as complete culture medium. In each experiment, parasite growth was partially synchronized by incubation in the absence of fetal calf serum for 24 to 48 h. Cells were then seeded at 0.5×10^6 to 1×10^6 cells per ml in complete medium and allowed to proliferate. Cell counts were performed with a Malassez hemocytometer after immobilization of cells with 1% formaldehyde in phosphate-buffered saline (PBS). Promastigote growth curves showed typical mid-log, late log, and stationary phases at days 3 and 4, 5, and 7, respectively. Samples of parasites were collected and washed three times with PBS, and various protein preparations were obtained, as described below.

Antileishmanial human sera. Sera were obtained from patients with acute leishmaniasis, from healed subjects, and from asymptomatic individuals with a positive DTH test (all subjects lived in the area of endemicity [16] mentioned above), as indicated in figure legends. Sera from subjects with a negative DTH reaction were used as controls.

Antigen preparations. Washed parasites were resuspended at 2×10^8 to 3×10^8 cells per ml in 10 mM Tris buffer (pH 8) containing 250 mM sucrose, 2 mM $MgCl_2$, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1,000 U of aprotinin per ml. These preparations were either stored at −20°C or immediately lysed for 4 h at 4°C in 0.5% Triton X-100 and centrifuged for 15 min at 15,000 × *g*, unless indicated otherwise. The protein content in the pellets (insoluble fraction) and supernatants was measured with the MicroBCA protein assay reagent kit (Pierce) as specified by the manufacturer. In functional assays (primed human peripheral blood mononuclear cell [PBMC] proliferation and IL-2 production [see below]), the parasites used as stimulating antigens were lysed by 11 cycles of freeze-thaw procedure in distilled water containing protease inhibitors. Then the whole lysate was separated into soluble and sedimenting fractions by 15,000 × *g* centrifugation for 10 min at 4°C. Nitrocellulose-bound proteins used in the functional assays were prepared as follows (adapted from the procedure in reference 20). Electrophoretically fractionated p14 and p18 antigens transferred onto the nitrocellulose sheet were identified by the labeling (see below) of outer peripheral lanes. Horizontal strips of nitrocellulose bounding p14 or p18 and control strips were cut appropriately ($l \times h$ = [5 × 1] mm^2 for microtiter plates, [10 × 1] mm^2 for 24-well plates) and washed quickly in a solution of 5,000 U of penicillin per ml and 5 mg of streptomycin per ml before being used for PBMC activation. Potential stimulatory activity of other nitrocellulose-bound proteins was tested on membrane sections of $l \times h = (2 \times 8)$ mm^2 for microtiter plates and (5 × 8) mm^2 for 24-well plates. The following sections of the membrane were tested: 21 to 28 kDa, 29 to 39 kDa, 40 to 62 kDa, and 63 to 102 kDa.

Nucleus preparations. Nuclei were prepared from stationary-phase promastigotes by the following method adapted from references 8 and 37. Washed cells (6×10^8) were lysed in 3 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–10 mM $MgCl_2$–2 mM dithiothreitol–250 mM sucrose buffer (nuclear homogenization buffer [NHB]) containing 0.5% Triton X-100 by 20 strokes in Dounce homogenizer and 10 passages through a 26-gauge needle. The lysate, in a final volume of 6 ml, was centrifuged for 20 min at 1,900 × *g*. The resulting pellet was washed in 6 ml of NHB, resuspended in 1 ml of the same buffer, and centrifuged at 100,000 × *g* for 90 min at 4°C over a 2 M sucrose cushion in NHB containing 0.5% Triton X-100. Cellular debris located at the NHB/sucrose interface were removed. The nuclear pellet was washed (for 15 min at 15,000 × *g*) in 50 mM HEPES–5 mM $MgCl_2$–2 mM dithiothreitol–0.1 mM EDTA containing 40% (vol/vol) glycerol and resuspended in 100 μl of the same buffer. The entire procedure was carried out on ice, and centrifugations were done at 4°C.

SDS-PAGE and Western blot analysis. Leishmanial antigens, prepared as described above, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14% polyacrylamide). The protein load per well is indicated in figure legends. The separated proteins were transferred onto nitrocellulose in 25 mM Tris–190 mM glycine–20% (vol/vol) methanol buffer at 15 V/cm for 30 min. The nitrocellulose strips were first saturated by incubation in PBS–1% (wt/vol) skim milk–2.5×10^{-4} M thimerosal (saturation buffer), incubated with sera diluted 1:10 in the same buffer for 4 h, and washed three times (5 min each) in PBS. Incubation of the nitrocellulose with the affinity-purified, Fc-specific, anti-human immunoglobulin G (IgG) peroxidase conjugate (Sigma) was carried out, at a 1:800 dilution in saturation buffer, for 2 h and was followed by three washes as above. Enzymatic activity was revealed with 1.5 mM diaminobenzidine–0.38 mM $CoCl_2$–0.03% H_2O_2 in PBS. The quality of the transfer of leishmanial proteins on the nitrocellulose was regularly checked and confirmed by concurrent gel staining with Coomassie blue and nitrocellulose membrane staining with Ponceau S.

Cross-blot assay. Anti-p14 and anti-p18 antibodies were immunopurified from human serum as follows. The insoluble cellular fraction of stationary promastigotes (corresponding to 10^8 cells per 6.4 cm of gel) was electrophoresed, transferred onto a nitrocellulose sheet, and incubated with the human serum, as described above. The sheet was washed, and the antibodies to the p14 and the p18 proteins were eluted from the corresponding horizontal strips with 70 μl of 200 mM glycine-HCl buffer (pH 2.8) for 1 min with vigorous shaking. The eluted antibodies were then neutralized with 4.5 μl of 1 M Tris, diluted in 400 μl of the saturation buffer, and used again in a Western blot experiment for immunological demonstration of proteins on a new nitrocellulose sheet.

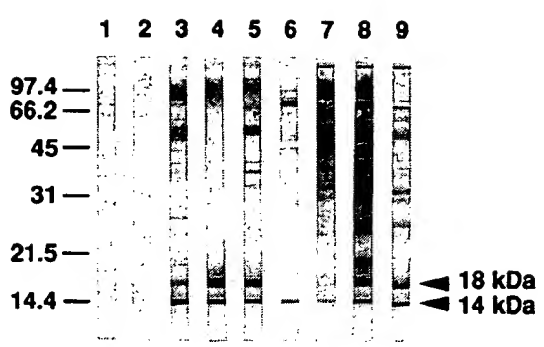

FIG. 1. Sera from subjects who have been exposed to *L. infantum* recognize p14 and/or p18 proteins on Western blot. Total proteins of *L. infantum* promastigotes were separated by SDS-PAGE (14% polyacrylamide) (55 μg of proteins per cm of gel) and transferred to nitrocellulose. Human sera, diluted 1:20, were incubated overnight with nitrocellulose strips. Sera were from leishmanin skin test-negative controls (lanes 1 and 2), leishmanin skin test-positive asymptomatic individuals (lanes 3 to 6), patients with healed leishmaniasis (lanes 7 and 8), and a patient with acute leishmaniasis (lane 9). Molecular mass markers are indicated.

PBMC. Peripheral blood was obtained from two patients healed of *L. infantum* infection (donors 2 and 3), from asymptomatic subjects with positive DTH tests and detectable anti-p14 and/or anti-p18 antibodies (donors 1, 4, and 5), and from leishmanin skin test-negative, control donors. PBMC were isolated by centrifugation of heparinized blood over lymphocyte separation medium (Eurobio, Les Ulis, France). The interface cells were washed three times in RPMI 1640 and resuspended at 1×10^6 to 1.5×10^6 cells per ml in complete culture medium. The cells were then seeded at 100 μl per well in flat-bottom culture plates for proliferation studies and at 1 ml per well in 24- or 48-well plates for IL-2 assays and were cultured at 37°C in a 5% CO_2, humidified atmosphere. Cell activation was initiated 16 to 24 h later, as described below.

Cell activation and proliferation assays. Cells were stimulated by (i) unfractionated promastigote antigens (15 μg/ml), (ii) insoluble (15 μg/ml) or (iii) soluble (15 μg/ml) antigen fractions prepared by freeze-thaw lysis and centrifugation, or (iv) electrophoretically fractionated nitrocellulose-bound antigens or were left unstimulated. Assays were carried out in triplicate in an incubation volume of 200 μl. Nitrocellulose fragments of the same surface excised after transfer from blank SDS-PAGE wells were used as controls. After 4 days of culture, cells were pulsed with 1 μCi of [^{3}H]thymidine per well, incubated for an additional 18 h, and harvested onto a fiberglass filter. Incorporated ^{3}H was measured by β counting. Data are represented as mean counts per minute ± standard deviation, in units of 10^3 cpm.

IL-2 assay. PBMC cultured in 1 ml in 24- or 48-well plates at 1×10^6 to 1.5×10^6 cells per well were activated as indicated above or left unstimulated and were incubated for an additional 36 to 42 h. The plates were stored at −20°C until cytokine production was measured. IL-2 levels in the supernatants were assayed by a sandwich enzyme-linked immunosorbent assay (ELISA), able to detect 10 pg of the cytokine per ml, as previously described (4).

Indirect immunofluorescence. Stationary-phase promastigotes were washed three times in ice-cold PBS, and 10 μl of a 10^6-cell-per-ml suspension was allowed to settle in 18-well immunofluorescence slides for 30 min at room temperature in a humidified atmosphere. Cells were fixed by incubation at 60°C and either stored at −20°C (up to 1 month) or used immediately. Parasites were incubated with control or tested sera at 1:10 × 2^n (n = 2 to 4) dilutions for 30 min at 37°C and then washed twice in PBS and once in distilled water. Antibody fixation was revealed with fluorescein isothiocyanate-conjugated anti-human IgG (Fluoline G; Biomérieux), prepared at 1:100 in 0.01% Evans blue for counterstaining.

RESULTS

Subcellular location of p14 and p18 proteins in stationary-phase promastigotes. Western blot analysis of the protein preparation from stationary-phase promastigotes typically shows several antigens, revealed by sera from treated patients and from subjects with a positive leishmanin skin test (Fig. 1) (17, 19). We observed, however, that some sera from asymp-

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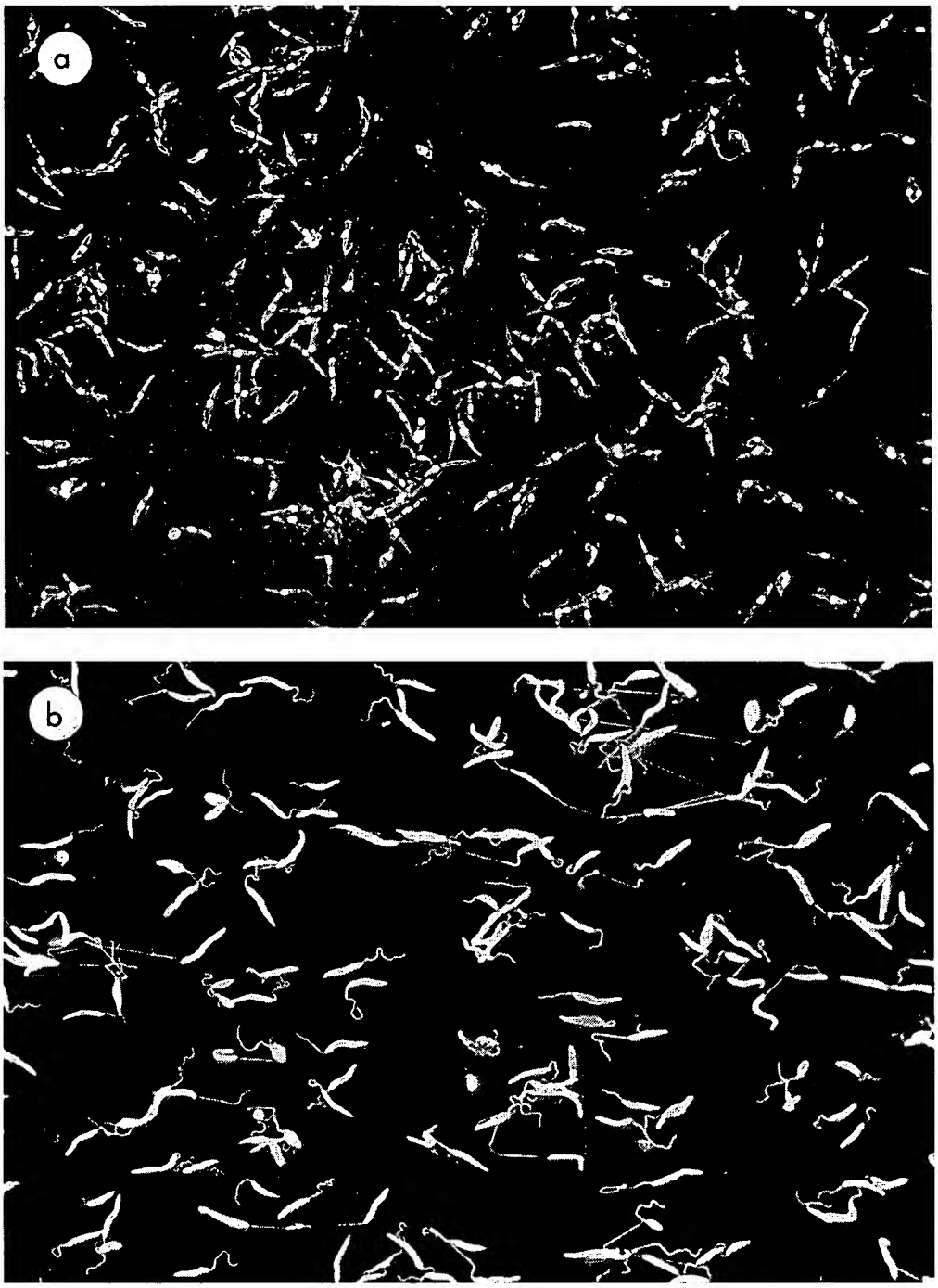

FIG. 2. Sera which detect mainly p14 and/or p18 antigens on Western blot stain promastigote nuclei in indirect immunofluorescence. (a) Fixed promastigotes were labeled with serum shown in Fig. 1, lane 4, which recognizes mainly p14 and p18 antigens, from a leishmanin skin test-positive asymptomatic individual and stained with fluorescein isothiocyanate-conjugated anti-human IgG. Red counterstaining was carried out with Evans blue; it allows one to visualize the promastigote outline. (b) Fixed promastigotes were labeled with serum shown in Fig. 1, lane 8, which recognizes multiple bands, from a patient with healed leishmaniasis and stained with fluorescein isothiocyanate-conjugated anti-human IgG. Magnification, ×400.

tomatic individuals exhibited a more restricted Western blot pattern, recognizing mainly p14 and/or p18 leishmanial proteins (Fig. 1, lanes 4 and 6). Interestingly, when analyzed by indirect immunofluorescence on whole parasites, these sera exhibited almost exclusively nuclear labeling (Fig. 2a); for comparison, a typical intense and homogenous IF staining of whole promastigotes, performed with a serum recognizing multiple bands on Western blot, is shown in Fig. 2b. This immunoflu-

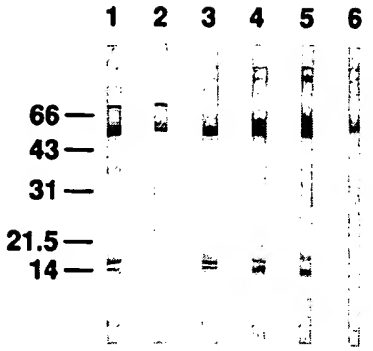

FIG. 3. Nuclei isolated from *L. infantum* stationary-phase promastigotes contain p14 and p18 antigens. Cellular fractions obtained in the process of preparation of nuclei, described in Materials and Methods, were analyzed by Western blotting. Lanes: 1, total parasite lysate of 2.5×10^6 cells; 2, supernatant from centrifugation at $1,900 \times g$ (2.5×10^6 cells); 3, corresponding pellet (2.5×10^6 cells); 4, pellet from ultracentrifugation over 2 M sucrose cushion at $100,000 \times g$ corresponding to nuclei of 10^7 cells; 5 and 6, pellets from centrifugation at $15,000 \times g$ of nuclei (lane 5; 5×10^7 cells) and of cellular debris located at the interface of sucrose/NHB (lane 6; 1.5×10^8 cells). Molecular mass markers are indicated.

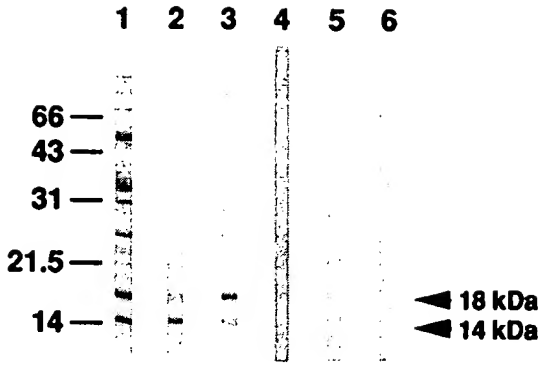

FIG. 4. p14 and p18 antigens share common epitopes. Anti-p14 and anti-p18 monospecific antibodies were immunopurified on nitrocellulose from the serum of the acutely ill patient (Fig. 1 lane 9), as described in Materials and Methods. Insoluble proteins were analyzed by Western blotting with the total serum (lane 1) and with anti-p14 (lane 2) and anti-p18 antibodies (lane 3). Control incubations in the absence of antigens (lane 4), of serum (lane 5), and of anti-IgG conjugate (lane 6) are shown. Molecular mass markers are indicated.

orescence pattern suggested that p14 and p18 antigens were located in parasite nuclei. Nuclear proteins were therefore prepared from stationary-phase promastigotes as described in Materials and Methods and analyzed by Western blotting. Figure 3 shows that, indeed, the nuclei isolated from the parasites contained p14 and p18 antigens (lanes 4 and 5), while no staining was detected in the remaining cellular fractions (lanes 2 and 6). An alternative procedure for nucleus preparation, modified from that in reference 22, gave the same result (not shown). We next confirmed that when the stationary-phase parasites were solubilized with 0.2% and up to 4% of Triton X-100, p14 and p18 proteins always remained in the pellet after $15,000 \times g$ centrifugation (not shown). Therefore, in what follows, Western blot analyses of p14 and p18 were carried out with pellets and supernatants of antigenic preparations obtained by solubilization of cells with 0.5% Triton X-100 and centrifugation at $15,000 \times g$. It should be added that disulfide bond disruption by 2-mercaptoethanol did not modify either the SDS-PAGE or the Western blot pattern, indicating a monomeric structure of p14 and p18.

Subcellular location of p14 and p18 proteins in logarithmically growing promastigotes. We next investigated whether p14 and p18 antigens were located exclusively in nuclei during all phases of parasite growth or whether a modulation of their expression and/or location could be detected. Preliminary experiments were carried out to determine the optimal protein load allowing a partial antigen quantification. For most tested sera, a 10-μg protein load of the insoluble fraction represented the threshold of p14 and p18 signal saturation and the ratio between the antigen detection and the signal saturation threshold was found to be 3:1 to 4:1 (data not shown). The expression of p14 and p18 during promastigote culture was then investigated, analyzing either a constant number of cells or a constant amount of proteins. We found that both antigens were again dominantly present in the insoluble fraction. Interestingly, both were also found in the solubilizable fractions but only on days 3 and 4 of the culture, corresponding to the mid-logarithmic growth phase (data not shown). These results indicate a transitory presence of p14 and p18 outside the nucleus in logarithmically growing promastigotes.

Antigenic relation between p14 and p18 proteins. Next, we carried out a cross-blot analysis of both proteins; monospecific anti-p14 and anti-p18 antibodies were obtained by immunopurification of human polyspecific, polyclonal antisera on nitrocellulose as described in Materials and Methods. Figure 4 shows that polyclonal antibodies directed against p14 antigen recognized p18 (lane 2) and, reciprocally, that antibodies directed against p18 antigen recognized p14 (lane 3), thus indicating that these proteins share a common epitope(s).

Carrying out experiments with the same antigen stock at different times after its preparation, we observed that p18 seemed to undergo a much faster degradation than p14 while stored at −20°C, as judged by noticeable fading of the p18 signal on Western blot. The causes of this phenomenon remain unclear: an autocatalytic activity or a p18-specific, cold-resistant catalytic activity can be evoked, since this phenomenon was slowed in the presence of protease inhibitors in the storage buffer and it did not occur when parasites were lysed directly in the electrophoresis sample buffer containing 3% SDS. We also found that p18 underwent a faster degradation than p14 at high pH. These observations will probably be explained as the role played by p14 and p18 proteins in the maturation of promastigotes becomes elucidated.

Activation of human primed T lymphocytes, induced in vitro by p14 and p18 proteins. Activation experiments were carried out on PBMC from five primed donors, described in Materials and Methods, and three negative controls. The complete proliferation data from the five tested subjects who have been exposed to *L. infantum* are compiled in Table 1 and presented as mean counts per minute ± standard deviation, in units of 10^3 cpm. First, the capacity of nitrocellulose-bound p14 and p18 to activate human primed PBMC was demonstrated: proliferation induced by p14 alone (donors 2, 3, 4, and 5) and p18 alone (donors 3, 4, and 5) or p14 plus p18 (donor 1) was, as expected, modest compared with that induced by phytohemagglutinin (PHA) at 1 μg/ml but was systematically higher than that of the control (blank nitrocellulose) for all primed PBMC which we examined. Next, the response to the insoluble fraction of leishmanial antigens (15 μg/ml), containing p14 and p18, was also analyzed and was compared with that induced by the soluble fraction (15 μg/ml) and by the unfractionated preparation (15 μg/ml). The data show that the response to the insoluble antigens was much stronger than that to the soluble

TABLE 1. Proliferation of human PBMC from subjects exposed to *L. infantum*[a]

Activator	Proliferation of PBMC (mean cpm ± SD) (10^3) from donor:				
	1	2	3	4	5
No activation	3.0 ± 0.6	1.1 ± 0.1	1.4 ± 0.4	0.1 ± 0.01	0.6 ± 0.2
Control nitrocellulose	5.2 ± 4.6	2.1 ± 0.1	3.1 ± 1.6	1.6 ± 0.7	4.0 ± 0.6
Nitrocellulose-bound p14	28.3 ± 4.2	15.4 ± 4.9	5.9 ± 2.6	4.0 ± 1.0	5.9 ± 0.5
Nitrocellulose-bound p18		ND[b]	4.6 ± 2.2	2.6 ± 0.4	8.2 ± 1.9
Insoluble fraction (15 μg/ml)	38.4 ± 2.8	ND	22.6 ± 0.3	ND	1.4 ± 0.1
Soluble fraction (15 μg/ml)	ND	ND	6.9 ± 0.4	5.1 ± 0.6	10.6 ± 3.8
Total lysate (15 μg/ml)	21.4 ± 6.8	14.3 ± 2.8	23.0 ± 2.7	2.0 ± 0.5	11.6 ± 5.8
PHA (1 μg/ml)	45.6 ± 9.1	66.1 ± 4.7	44.1 ± 2.0	191.8 ± 31.5	139.9 ± 13.3

[a] Control donors were subjects who had not been exposed to *L. infantum*; no proliferation was induced by leishmanial antigens; PHA-induced proliferation, expressed as mean counts per minute ± standard deviation, in units of 10^3 cpm, was 41.6 ± 8.7, 74.2 ± 11.7, and 91.7 ± 7.4 for three different donors.
[b] ND, not determined.

fraction for one donor (donor 3) while it was much lower for another donor (donor 5). The stimulatory activity of other membrane-bound proteins was examined in some experiments (donors 3, 4, and 5 [data not shown]). We found that only one donor (donor 3) responded significantly and that this response was only to antigens bound on nitrocellulose between 29 and 39 kDa. No proliferation was induced by other membrane-bound proteins (the sections of the membrane tested were 21 to 28 kDa, 29 to 39 kDa, 40 to 62 kDa, and 63 to 102 kDa). The quality of the transfer on the nitrocellulose was, of course, checked by gel staining with Coomassie blue and membrane staining with Ponceau S. In all experiments, PBMC obtained from negative controls (as assessed by negative skin test and Western blot pattern) were included and were shown to respond to stimulation by PHA (see the footnote to Table 1) but not to stimulation by the tested preparations of leishmanial antigens.

In a second series of experiments, PBMC from two positive donors (donors 2 and 5) and of two control individuals were examined in IL-2 assays. Figure 5 shows that the IL-2 production elicited by the whole promastigote lysate and by nitrocellulose-bound p14 (donors 2 and 5) and p18 (donor 5) was again small but significant compared with control stimulation (no activation or blank membrane). Control PBMC obtained from negative individuals did not respond to stimulation by leishmanial antigens. The IL-2 response to PHA of all tested donors is described in the legend to Fig. 5.

DISCUSSION

In this article, we report on a partial parasitological and antigenic characterization of two proteins from *L. infantum* promastigotes, p14 and p18, and on an in vitro analysis of their potential to activate in vivo primed human T cells.

The interesting feature of these proteins which prompted our study was the finding of a significant correlation between the humoral response elicited by these proteins and a positive leishmanin DTH reaction, testifying to the occurrence of cell-mediated immunity. Sera from patients with classic visceral leishmaniasis (active or healed) contain antibodies, shown to exhibit high specificity for *L infantum*, directed against p14 and p18 (19). Furthermore, anti-p14 and/or anti-p18 antibodies are quite systematically detectable in sera of asymptomatic individuals who live in areas where *L. infantum* infection is endemic and have a positive leishmanin skin test (17). Finally, the Western blot profile rich in antigenic bands revealed by sera from acutely ill patients (compatible with high levels of parasite-specific antibodies developed early in disease) contrasts with the detection predominantly of p14 and p18 by sera from asymptomatic individuals (18). Taken together, these observations suggested that the humoral response elicited by p14 and p18 is strong and long lasting. We thus attempted to determine whether these proteins might also contribute to the cell-mediated immunity.

First, we studied the subcellular location of p14 and p18 antigens and investigated whether their expression varied as a function of the parasite growth in vitro. Indeed, the transformation of log-phase promastigotes into stationary phase correlates with events occurring naturally during maturation of the parasite: it was demonstrated that promastigotes logarithmically growing in the culture medium mimic the procyclic stage of development inside the sand fly gut, while the station-

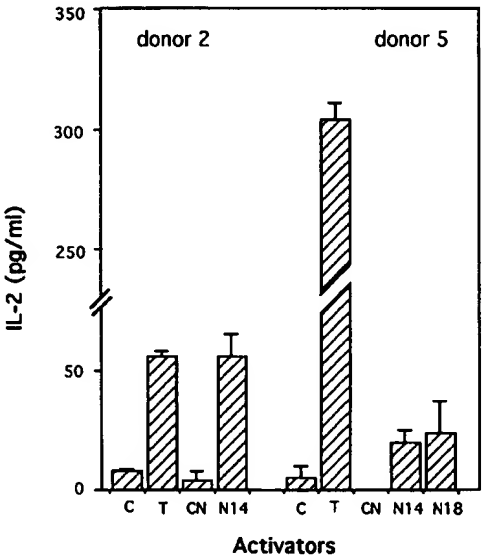

FIG. 5. Nitrocellulose-bound p14 and p18 proteins stimulate IL-2 production by primed human T lymphocytes. PBMC were from donor 2 (healed patient) and donor 5 (asymptomatic, leishmanin DTH-test-positive subject). Cells (10^5 cells per well) were stimulated by nitrocellulose-bound p14 (N14; donors 2 and 5) or p18 (N18; donor 5) or by total promastigote lysate (T; 15 μg/ml). Control incubations were carried out in the absence of activators (C) or in the presence of a blank nitrocellulose fragment (CN). IL-2 production was measured by ELISA after 36 h (donor 2) or 46 h (donor 5) of cell stimulation. The level of PHA-induced IL-2 was 800 ± 48 pg/ml in donor 2 (PHA at 20 μg/ml) and 3.90 ± 0.13 ng/ml in donor 5 (PHA at 75 μg/ml). No IL-2 production was induced by leishmanial antigens in PBMC of two tested control subjects who were not exposed to *L. infantum*; the PHA-induced (50 μg/ml) IL-2 levels in these donors were 4.13 ± 0.6 and 1.14 ± 0.5 ng/ml.

ary phase of the culture corresponds to the metacyclic, infective form of the parasite (30–32). We showed, by immunofluorescent staining and through a Western blot study of subcellular fractions, that in the stationary phase of promastigote growth, p14 and p18 proteins were located only in the parasite nuclei. In the logarithmically growing parasites, p14 and p18 were still present predominantly in the nuclei, but their transitory expression out of the nucleus, in the middle of the log phase, was detected. We also found that despite a 1.5-fold reduction of total protein synthesis as promastigotes approach the metacyclic growth stage (our results, data not shown; 1), the percentage of p14 and p18 expressed by the parasites increased (data not shown). These results indicate that the expression of p14 and p18 proteins was modulated while promastigotes proliferated in vitro and suggest that it might be regulated developmentally during natural maturation in the phlebotomine vector.

The common location of the p14 and p18 antigens and their concurrent evolution during promastigote culture prompted us to inquire whether they could be antigenically related. We found that immunopurified antibodies directed against p14 (or against p18) reacted with nitrocellulose-bound p18 (or p14), pointing to a likely common epitope(s). Further characteristics of p14 and p18 proteins in the promastigote and amastigote stages of *L. infantum*, the mechanisms of their intracellular trafficking, and a possible relationship between p14 and the recently cloned histone H3 of *L. infantum* (36) are at present under investigation in our laboratory.

Next, we analyzed the in vitro activation of human primed PBMC. We studied cell proliferation induced by leishmanial proteins in five positive donors and IL-2 production in two positive donors.

The proliferative responses induced by the unfractionated preparation of leishmanial antigens, by its soluble fraction, and by antigens sedimenting at 15,000 × *g* were measured. The finding that the insoluble proteins induced a strong proliferation, comparable to that induced by whole lysate in some primed individuals (donors 1 and 3), is important, since in most published reports the stimulatory activity of the soluble fraction only was examined. We also performed a (rough) quantitation of the transferred proteins by Ponceau S staining of the membrane after simultaneous transfer of the whole lysate at various dilutions and of molecular weight standards at known concentrations (results not shown). We estimated that p14 and p18 each account for 0.05 to 1% of the total transferred proteins and thus might represent up to 4% of the insoluble proteins. In these functional experiments, insoluble and soluble fractions were obtained after freeze-thaw lysis. Therefore, the separation is less complete than after Triton X-100 cell lysis, since freeze-thaw disruption of parasites is rarely perfect, even after 11 cycles. We found in control experiments that some proteins, including p14 and p18, could be detected by Western blot in both the pellet and the supernatant after the freeze-thaw disruption, in contrast with Triton X-100 cell lysis. These results indicate that the response to the whole lysate was, in fact, due to the nuclear antigens in some individuals.

We demonstrated directly that p14 and p18 in vitro had the capacity to activate PBMC from sensitized individuals. Cell proliferation was specifically stimulated by nitrocellulose-bound p14 and p18 in all tested positive donors, and IL-2 production was found in two donors. It should not be astonishing that activation induced by p14 and p18, which stimulate their specific memory cells, was relatively low compared with that induced by PHA, a potent polyclonal activator. The respective contributions of p14 and p18 to PBMC activation seemed equivalent. Even if the values were slightly different for different donors, the differences did not appear significant, and, obviously, we cannot say whether these variations might arise from individual major histocompatibility complex antigens and/or from the T-cell repertory.

The capacity of membrane-bound p14 and p18 to activate all of the tested human PBMC sensitized in vivo contrasts with a lack of stimulatory activity of other membrane-bound proteins. Among three donors examined (donors 3, 4, and 5), we found that only one (donor 3) responded significantly and then only to the antigen(s) bound on nitrocellulose between 29 and 39 kDa. No proliferation was induced by other membrane-bound proteins. Taken together, our results suggest that an antigenic determinant(s), dominant in some individuals, might exist on both proteins.

Data presented in this paper do not permit us to determine whether the in vitro p14- and p18-induced T-cell responses are protective in vivo. Specifically induced proliferation and IL-2 production in primed PBMC point to a possible existence of a p14- and p18-specific Th1-type response; the presence of humoral immunity provides indirect evidence for in vivo expansion of the Th2 subset. Thus, p14- and p18-specific Th1- and Th2-type responses could coexist in human visceral leishmaniasis. The role and significance of the humoral response in leishmaniasis remain unknown. As the parasite induces an abundant production of antibodies, it is possible that the protective cell-mediated immunity becomes less efficient (2), perhaps as a result of a competition between T- and B-cell repertoires. A study of a cutaneous leishmanial infection in a murine model suggested that the presence of B lymphocytes but not of antibodies was required for the generation of an effector T-cell population which, however, seemed unrelated to delayed hypersensitivity (34). Alternatively, it is possible to imagine that coexistence of Th1-like and Th2-like responses might contribute to the balance established between the human host and its persistent *Leishmania* parasite, with the Th2 subset preventing the Th1 population from undergoing immoderate expansion.

Specific antigens that stimulate T lymphocytes from individuals with immunity to leishmaniasis are of particular interest, since they may be potential candidates for a vaccine. Only a few have been characterized. The properties of the major surface protease gp63 and of lipophosphoglycan are by far the best described (3, 5, 26). It has been shown not only that gp63 was a powerful stimulator of proliferation and of IFNγ production in T cells from cured patients but also that the native gp63 could effectively induce T-cell responses by in vitro immunization of normal PBMC (27). Some host-protective T-cell epitopes of gp63 were identified in mice (9, 38) and in humans (28). T-cell-activating properties of lipophosphoglycan first remained a subject of debate. Then a protein complex which copurified with lipophosphoglycan, the lipophosphoglycan-associated protein, was shown to account for in vitro T-cell activation in murine (10) and human (14, 29) systems. Leishmanial gp30 and gp42 antigens were identified by Western blotting with patient sera and were found to stimulate in vitro human lymphocytes (23). Recently, a 36-kDa protein, expressed in promastigote and amastigote stages of *L. major*, was cloned (21); it was selected by screening of an expression library of *L. major* for the identification of major histocompatibility complex class II-associated antigens and was recognized by a protective murine Th1 clone. A 24-kDa recombinant portion of this antigen was used together with IL-12 to protect susceptible BALB/c mice (21). Interesting features were demonstrated by a previous immunoblotting analysis of T-cell activation by fractionated leishmanial antigens (20). The authors found that specific T-cell responses were elicited by a very

large number of leishmanial proteins in immune (healed) individuals whereas PBMC of patients with nonhealing lesions responded to a very limited number of antigens. No obvious immunodominant antigen was found in this quite extensive analysis. The control and resolution of infection in humans might necessitate sensitization to several antigens, including p14 and/or p18. Experiments are in progress to further characterize both proteins and to determine their potential immunoprotective capacities.

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inflammation around pulmonary blood vessels and airways. Moreover, high levels of Th2-associated cytokines (IL-4 and IL-5) were generated when lung-draining lymph node and tissue cells were restimulated with L. major %%%lysate%%%. These data suggest that the lung environment per se favors Th differentiation towards the Th2 phenotype.

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Evaluation of recombinant K39 (rK39) antigen ELISA in the diagnosis of infantile visceral %%%Leishmaniasis%%% in South-West Saudi Arabia
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ABSTRACT: Recombinant K39 (rK39) and %%%Leishmania%%% donovani (Ld) %%%lysate%%% enzyme-linked immunosorbent assays (ELISA) detected high levels of anti-%%%Leishmania%%% specific IgG antibodies in infantile visceral %%%leishmaniasis%%% (VL) in Saudi Arabia. The mean optical density (OD) level of the anti-rK39 antibodies (2.113 +- 0.104) was significantly higher than the mean OD level of anti-Ld %%%lysate%%% antibodies (1.432 +- 0.082) ($p < 0.0001$). The sensitivity and specificity of rK39 and Ld %%%lysate%%% ELISA in detecting VL were 100% when comparing VL patients to normal endemic controls. rK39 ELISA was more specific than Ld %%%lysate%%% ELISA in identifying true VL from other coendemic infections like malaria and brucellosis (92.3%, 76.9%, respectively). rK39 antigen did not react with auto-reactive antibodies in autoimmune systemic lupus erythematosus (SLE) and was more specific than Ld %%%lysate%%% antigen in identifying anti-%%%Leishmania%%% specific antibodies from auto-reactive autoimmune antibodies. This suggests that rK39 ELISA has a good potential for sensitive and specific diagnosis of infantile VL in Saudi Arabia.

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Immunoglobulin subclass distribution and diagnostic value of %%%Leishmania%%% donovani antigen-specific immunoglobulin G3 in Indian kala-azar patients
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Immunoglobulin Subclass Distribution and Diagnostic Value of *Leishmania donovani* Antigen-Specific Immunoglobulin G3 in Indian Kala-Azar Patients

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Visceral leishmaniasis, or kala-azar, a fatal tropical disease, remains problematic, as early diagnosis is difficult and treatment often results in drug resistance and relapse. We have developed a sensitive enzyme-linked immunosorbent assay (ELISA), using leishmanial membrane antigenic extracts (LAg) to detect specific antibody responses in 25 untreated Indian visceral leishmaniasis patients. To investigate the pathogenetic significance of isotype markers in kala-azar, relative levels of specific immunoglobulin G (IgG), IgM, IgA, IgE, and IgG subclasses were analyzed under clinically established diseased conditions. Since LAg showed higher sensitivity for specific IgG than lysate, the immunoglobulin isotype responses were evaluated, with LAg as antigen. Compared to 60 controls, which included patients with malaria, tuberculosis, leprosy, and typhoid and healthy subjects, visceral leishmaniasis patients showed significantly higher IgG (100% sensitivity, 85% specificity), IgM (48% sensitivity, 100% specificity), and IgE (44% sensitivity, 98.3% specificity) responses. Low levels of IgA in visceral leishmaniasis patients contrasted with a 13-fold-higher reactivity in sera from patients with leprosy. Among IgG subclasses, IgG1, -3, and -4 responses were significantly higher in visceral leishmaniasis patients than in the controls. IgG2 response, however, was significantly higher (twofold) in leprosy than even visceral leishmaniasis patients. The rank orders for sensitivity (IgG = IgG1 = IgG3 = IgG4 > IgG2 > IgM > IgE > IgA) and specificity (IgM = IgG3 > IgE > IgG4 > IgG2 > IgG > IgG1 > IgA) for LAg-specific antibody responses suggest the potentiality of IgG3 as a diagnostic marker for visceral leishmaniasis.

Human visceral leishmaniasis, kala-azar, is a tropical disease caused by the protozoan parasites of the *Leishmania donovani* complex. The parasites multiply in the macrophages of the spleen, liver, bone marrow, and lymph nodes, resulting in a progressive disease which is invariably fatal if untreated. Infection by *L. donovani* in humans induces T-cell anergy as assessed by the depression of delayed-type hypersensitivity reaction and failure of peripheral blood T cells to proliferate (18, 19) and to produce gamma interferon (IFN-γ) and interleukin (IL)-2 in response to *Leishmania* antigens (8, 11). Cytokine analysis reveals enhanced induction of IFN-γ, IL-10, and/or IL-4 mRNA in tissues (16, 23), and the enhanced presence of IL-4 in circulation (40) of kala-azar patients. While the presence of these cytokines suggests a coexistence of Th-1- and Th-2-like responses in the clinical stage of the disease, the absence of IL-2 points to the dominance of the Th-2 response. The disease is also characterized by high levels of *Leishmania*-specific antibodies (3). Since cytokines elaborated by activated T cells are required for the regulation of isotype switch during B-cell development (15, 24, 37), a study of the subclass distribution of the antibodies may shed new light on the processes involved in the polarization of the immune responses during disease. Leishmanial membrane antigens of *L. donovani* (LAg) have been effectively used to investigate immunological responses during disease progression in murine models of visceral leishmaniasis (2). Herein, we report the subclass distribution and the fine specificity of the antibody response to LAg in the sera of Indian kala-azar patients.

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MATERIALS AND METHODS

Study subjects. The subjects of the present investigation were 25 Indian patients with visceral leishmaniasis admitted to School of Tropical Medicine, Calcutta, India. These patients came from Bihar (eastern India), one of the main areas of endemicity. Diagnosis of these patients was confirmed parasitologically by the demonstration of *Leishmania* amastigotes in spleen and/or bone marrow aspirates. Blood was obtained after diagnosis, before the initiation of chemotherapy. Sixty individuals included as controls consisted of 15 malaria patients infected with *Plasmodium falciparum* or *Plasmodium vivax* or both, 10 typhoid patients, 15 tuberculosis patients, 8 leprosy patients, and 12 healthy controls from the Indian Institute of Chemical Biology (IICB). The endemic diseases were confirmed bacteriologically in the case of typhoid, tuberculosis, and leprosy and parasitologically in the case of malaria, and sera were collected before treatment.

Preparation of antigen. *L. donovani* AG83, originally isolated from an Indian kala-azar patient, was cultured in vitro for antigen preparation as described earlier (1). Briefly, stationary-phase promastigotes, harvested after the third or fourth passage, were washed four times in cold phosphate-buffered saline (PBS) (pH 7.2) and resuspended at a concentration of 1.0 g of cell pellet in 50 ml of cold 5 mM Tris-HCl buffer, pH 7.6. The suspension was vortexed and centrifuged at 2,310 × *g* for 10 min. The crude ghost membrane pellet thus obtained was resuspended in the same Tris buffer and sonicated in an ultrasonicator. The suspension was centrifuged at 4,390 × *g* for 30 min, and the supernatant containing the LAg was harvested and stored at −70°C until use. The amount of protein obtained from 1.0 g of cell pellet, as assayed by the method of Lowry et al. (26), was 16 mg. The lysate used in this study was prepared from 5×10^7 stationary-phase promastigotes per ml according to the method of Jaffe and Zalis (21). Protein concentration (5 mg/ml) was assessed as described above.

Enzyme-linked immunosorbent assay (ELISA). For serological studies, microtiter plates (Tarsons) were coated overnight with 2 μg of lysate or LAg per well. For *Leishmania*-reactive immunoglobulin G (IgG), IgM, IgA, and IgE antibody determination, the antigen-coated plates were incubated with sera diluted 1:1,000-fold, and reacted with peroxidase-conjugated goat anti-human IgG, IgM, IgA, and IgE polyclonal antibodies (Sigma Immunochemicals) at a 1:5,000 dilution and developed with *o*-phenylenediamine dihydrochloride (1). For IgG subclass determination, human sera were reacted with mouse anti-human IgG1,

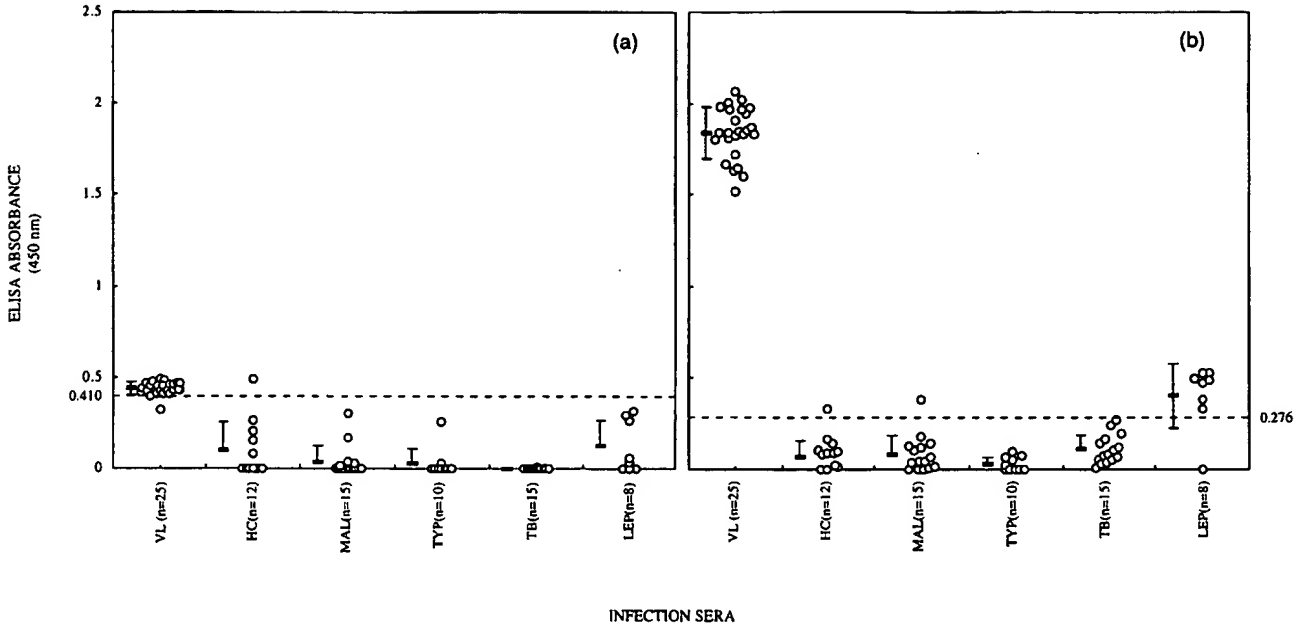

FIG. 1. Dot plots showing specific IgG responses (absorbance values) of sera from healthy controls and patients with visceral leishmaniasis, malaria, typhoid, tuberculosis, and leprosy to leishmanial lysate (a) and LAg (b). The horizontal bars represent the means ± standard deviations for the different groups. The dotted line indicates the cutoff value (mean of healthy controls + 2 standard deviations).

IgG2, IgG3, and IgG4 monoclonal antibodies (1:3,000 dilution; Sigma Immunochemicals). Bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (1:5,000 dilution; Sigma Immunochemicals) (1).

Statistical analysis. All data comparisons were tested for significance by using Student's *t* test; *P* values of < 0.05 were considered significant. The lower limit of positivity (cutoff) was determined by the mean of healthy controls + 2 standard deviations (13, 14).

RESULTS

Serum IgG specificity for *L. donovani* lysate and LAg. Reactivities of serum IgG antibodies of kala-azar patients to the parasite lysate were compared to those of LAg. At a 1:1,000 dilution of sera, 20 of 25 patients were positive for the lysate, with IgG absorbance values ranging from 0.319 to 0.493 (Fig. 1a). Reactivity with LAg, however, resulted in 100% sensitivity, with significantly higher IgG absorbance values (1.517 to 2.066; Fig. 1b). Serum specimens from patients with diseases such as malaria, typhoid, tuberculosis, and leprosy were negative for the lysate, and only 1 of 12 serum samples from normal control individuals analyzed was found to be positive (Fig. 1a). Conversely, 1 of 12 healthy controls and 1 of 15 malaria patients were positive, while all typhoid and tuberculosis serum specimens were negative for LAg (Fig. 1b). The highest cross-reactivity was observed with sera from leprosy patients (seven of eight samples). However, the mean ± standard deviation of IgG absorbance (0.403 ± 0.176) of these specimens was just above the cutoff value of 0.276 and significantly lower than the mean IgG response observed with kala-azar patient sera. Since antibody reactivities of sera from kala-azar patients with LAg were higher than with lysate and 100% sensitive, Ig subclass distribution analysis was restricted to LAg.

LAg-specific serum Ig antibodies. Antibody reactivities of IgG, IgM, and IgE of sera from patients with visceral leishmaniasis with LAg were significantly higher than those of normal controls and those of patients with other diseases such as malaria, typhoid, tuberculosis, and leprosy ($P < 0.05$; Table 1). The IgA reactivity with LAg was, however, predominant in sera from patients with leprosy, with titers 13-fold higher than those even of patients with visceral leishmaniasis.

TABLE 1. Comparison of the serological responses of visceral leishmaniasis to other diseases

Serogroup[b]	Antibody level[a]				No. of patients
	IgG	IgM	IgA	IgE	
VL	1.839 ± 0.143[c]	0.878 ± 0.455[c]	0.039 ± 0.084	0.083 ± 0.043[c]	25
HC	0.094 ± 0.091	0.250 ± 0.295	0.051 ± 0.93	0.022 ± 0.035	12
MAL	0.085 ± 0.100	0.140 ± 0.130	0.002 ± 0.007	0.003 ± 0.004	15
TYP	0.031 ± 0.037	0.158 ± 0.205	0.091 ± 0.162	0	10
TB	0.110 ± 0.078	0.315 ± 0.281	0.158 ± 0.189	0	15
LEP	0.403 ± 0.176[c]	0.249 ± 0.246	0.518 ± 0.312[c]	0.006 ± 0.011	8
Total					85

[a] Absorbance values (means ± standard deviations).
[b] VL, visceral leishmaniasis; HC, healthy controls; MAL, malaria; TYP, typhoid; TB, tuberculosis; LEP, leprosy.
[c] Significantly different ($P < 0.05$) from the value for healthy control group as calculated by Student's *t* test.

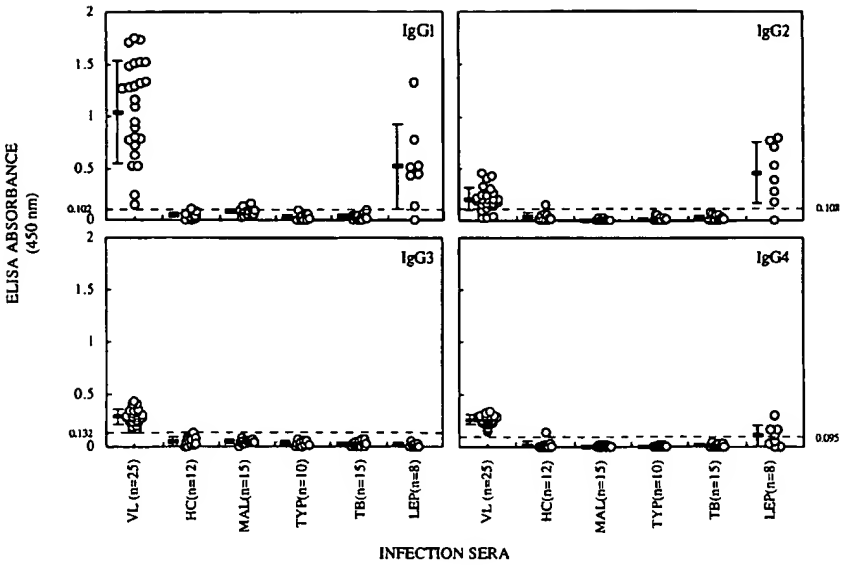

FIG. 2. LAg-specific antibody reactivities of IgG subclasses (absorbance values) of individual sera from healthy controls and patients with visceral leishmaniasis, malaria, typhoid, tuberculosis, and leprosy. The horizontal bars represent the means ± standard deviations for the different groups. The dotted line indicates the cutoff value (mean of healthy controls + 2 standard deviations).

Antigen-specific distribution of serum IgG subclasses. Since the analysis of sera from patients with kala-azar revealed high levels of IgG antibody response to LAg, the IgG subclass specificity was further examined. The results demonstrate that serum samples from all 25 visceral leishmaniasis patients were positive for IgG1, IgG3, and IgG4 antibodies, whereas 21 of 25 had antibodies of the IgG2 subclass (Fig. 2). All these IgG subclasses showed significantly higher reactivity with LAg than normal controls with the dominance of IgG1, in agreement with a previous report (38). While LAg-specific reactivities of all the IgG subclasses were minimal with sera from patients with other diseases, sera from patients with leprosy showed significant levels of antibody responses. Seven of eight samples tested had IgG1 and IgG2 subclasses, and four had IgG4. Moreover, the mean IgG2 response in sera from patients with leprosy was twofold higher even than that of patients with visceral leishmaniasis (Fig. 2). Surprisingly, however, samples of neither leprosy nor any disease other than kala-azar had LAg-specific IgG3 subclass antibodies. Table 2 summarizes the sensitivity and specificity of IgG, IgM, IgE, and IgG subclasses of sera from patients with visceral leishmaniasis.

TABLE 2. Percent sensitivity and specificity of IgG, IgM, IgA, IgE, and IgG subclasses in visceral leishmaniasis patients

Antibody	Sensitivity[a] (%)	Specificity[a] (%)
IgG	100	85.0
IgM	48	100.0
IgA	4	80.0
IgE	44	98.3
IgG1	100	83.3
IgG2	84	86.6
IgG3	100	100.0
IgG4	100	91.6

[a] Calculations were done with respect to the ELISA cutoff values of 0.276, 0.840, 0.237, 0.092, 0.102, 0.108, 0.132, 0.095 optical density for IgG, IgM, IgA, IgE, IgG1, IgG2, IgG3, and IgG4, respectively.

DISCUSSION

We found that while a high proportion of Indian kala-azar patients have elevated levels of anti-LAg IgG, IgM, IgE, and IgG subclass antibodies, IgG, IgG1, IgG3, and IgG4 were present in sera from all the patients, with IgG3 being specifically associated with this disease. Although investigations in murine models of *Leishmania major* and *L. donovani* infections clearly demonstrate a Th-2/IL-4/IgG1 relationship with disease progression, and a Th-1/IFN-γ/IgG2a relationship with resistance and protective immunity (1, 2, 4), such a relationship in humans is not fully understood. An association between antibody isotypes, cytokine profiles, and pathogenesis has been made for some diseases such as leprosy (12, 20), AIDS (6, 27), lymphatic filariasis (25), onchocerciasis (32), and malaria (28). In American cutaneous leishmaniasis, strong cell-mediated immunity and the predominance of IgG1, IgG2, and IgG3 isotypes in localized cutaneous and mucocutaneous leishmaniasis have been linked with Th-1 reactivity, whereas IgG4 subclass antibody response in sera of diffuse cutaneous leishmaniasis patients has been correlated with a Th-2 cell response (7, 9, 29, 35). Cytokine analysis of human visceral leishmaniasis suggests that the Th-2 response will be stronger than the Th-1 response during the active phase of the disease (8, 16, 23, 40). Investigations of IgG subclass response during disease show significant stimulation of all the IgG subclasses in Sudanese patients, with higher levels of IgG3 and IgG4 than IgG1 (13). Conversely, Venezuelan patients have a dominant IgG1 response followed by IgG4 (38). Indian kala-azar patients also showed a predominant IgG1 subclass antibody response, but the levels of IgG3, IgG4, and IgG2 were also significant. These subclasses of human IgG are endowed with unique biological and functional properties, including their response to different types of antigens (22). The elicitation of IgG1, IgG3, and IgG4 antibodies in kala-azar sera may be due mostly to the presence of protein antigens, and the elicitation of IgG2 antibodies in kala-azar sera may be due mostly to the presence of carbohydrate antigens, as reported for viral, bacterial, and parasitic infections (6, 12, 20, 25, 32). Induction of IgG1 and IgG2 is

IFN-γ dependent, and IgG3 and IgG4 depend on IL-4 and are down regulated by IFN-γ (15, 24). The elevation of IFN-γ in kala-azar patients (23, 40) and the strong reactivity of IgG1 during disease appear to be consistent with the above observations. Their presence, however, fails to control the infection. The absence of IL-2, a Th-1 mediator, suggests a lack of Th-1 response during disease (8, 11). Stimulation of serum IgG3 and IgG4 during infection, together with the expression of cytokines such as IL-10 and IL-4 (16, 40), which are also responsible for the upregulation of these IgG subclasses, provides further evidence in support of a Th-2 cell response in determining the outcome of the disease. One explanation for the presence of IgG1 in kala-azar patients may be due to IFN-γ derived from alternative cell sources such as natural killer cells and γδ T cells (10, 39).

LAg-associated serological responses of patients with diseases other than kala-azar were observed to be maximal for leprosy for all isotypes except IgM, IgE, and IgG3. In contrast to a previous report of low reactions of leprosy sera with soluble extracts of *Leishmania* promastigote antigen for all isotypes (38), LAg gave strong reactions with IgG, IgA, IgG1, and IgG2 and low reactivity with IgG4. Further, reactions with IgA and IgG2 were 13- and 2-fold higher, respectively, than even kala-azar patient sera. Leprosy sera show reactivity with lipoarabinomannan B (LAM), a carbohydrate component of *Mycobacterium leprae*, through IgG2 and IgG4 and rarely with IgG3 (12). Phenolic glycolipid (PGL-1), another cell wall carbohydrate of *M. leprae*, reacts strongly with IgA (31) and IgG1 (12) antibodies in leprosy sera. While it is not understood how antibodies in leprosy sera react with LAg, these observations point to cross-reacting epitopes of LAM and PGL-1 in *L. donovani* LAg.

Amongst all the Ig isotypes and IgG subclasses studied, only IgG3 showed 100% sensitivity and specificity for LAg in visceral leishmaniasis patients. Hence, IgG3 antibody may be a more specific marker for this disease than IgG, which shows low cross-reactivity with other diseases and significant reactivity with leprosy. Moreover, we have found that although there is a decline in the levels of IgG and its subclasses after successful treatment, the decrease is maximal in IgG3 (data not shown), suggesting that IgG3 may be a useful tool for diagnosis as well as for the prognosis of visceral leishmaniasis. IgG3 elevation during leishmaniasis was reported earlier (13, 29, 34), and high specificity and sensitivity for IgG3 have been found in Sudanese visceral leishmaniasis patients (13). Better sensitivity and specificity for IgG3 observed in our studies may be due largely to the specificities of the antibodies to the antigen studied (35) in addition to ethnic variation and differences in parasite genotypes. The significance of IgG3 specificity in visceral leishmaniasis is not clearly understood. IgG3 in malaria is associated with recovery from the fatal disease (33), and skewing of the response toward the IgG3 subclass is merozoite surface antigen 2 specific (30). In leprosy, disease progression is correlated with selective increases in IgG3, along with IgG1 responses (20). Another example of antibody response which is significantly skewed toward IgG3 is the response to the outer membrane protein of *Branhamella catarrhalis* (17). While there are reports of involvement of T-cell-derived cytokines in the regulation of switch factors for IgG3 (5, 24), little is known about the factors which may preferentially induce the production of IgG3 in humans. Functionally, IgG3 is considered to be the most effective subclass for activating the complement pathway (22) and is known to mediate cell lysis by monocytes or Fc receptor-bearing lymphocytes (36). However, the protective role of leishmania-specific antibodies in human visceral leishmaniasis is still controversial. In conclusion, our serological data demonstrate the potentiality of LAg as an important antigen in the diagnosis of the outcome of infection with *L. donovani*, with IgG3 as a marker for the identification of individuals with visceral leishmaniasis.

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ABSTRACT: IL-12 is a pluripotent cytokine that interacts with NK and T cells to play a central role in the initiation and maintenance of Th1 responses and IFN-gamma production. Because of the interactive relationship between IL-12 and IFN-gamma response to infectious

Characterization of a Leishmania tropica Antigen that Detects Immune Responses in Desert Storm Viscerotropic Leishmaniasis Patients

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Medical Sciences

Characterization of a *Leishmania tropica* antigen that detects immune responses in Desert Storm viscerotropic leishmaniasis patients

(parasite/diagnosis/repetitive epitope/subclass)

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ABSTRACT **A chronic debilitating parasitic infection, viscerotropic leishmaniasis (VTL), has been described in Operation Desert Storm veterans. Diagnosis of this disease, caused by *Leishmania tropica*, has been difficult due to low or absent specific immune responses in traditional assays. We report the cloning and characterization of two genomic fragments encoding portions of a single 210-kDa *L. tropica* protein useful for the diagnosis of VTL in U.S. military personnel. The recombinant proteins encoded by these fragments, recombinant (r) Lt-1 and rLt-2, contain a 33-amino acid repeat that reacts with sera from Desert Storm VTL patients and with sera from *L. tropica*-infected patients with cutaneous leishmaniasis. Antibody reactivities to rLt-1 indicated a bias toward IgG2 in VTL patient sera. Peripheral blood mononuclear cells from VTL patients produced interferon γ, but not interleukin 4 or 10, in response to rLt-1. No cytokine production was observed in response to parasite lysate. The results indicate that specific leishmanial antigens may be used to detect immune responses in VTL patients with chronic infections.**

Infection by the parasite *Leishmania* can result in a broad spectrum of pathological outcomes in the human host, ranging from simple self-healing cutaneous lesions to acute visceral leishmaniasis (VL), commonly referred to as kala-azar. The differing pathologies usually correlate with infection by differing species. *Leishmania donovani* and *Leishmania infantum* usually cause VL, with symptoms including fever, emaciation, hypergammaglobulinemia, hepatosplenomegaly, and pancytopenia. *Leishmania major* and *Leishmania tropica* generally cause cutaneous leishmaniasis (CL). Exceptional cases have been described, such as visceral outcomes in individuals infected with *L. tropica* (1, 2).

More recently, exposure of U.S. soldiers to *L. tropica* has resulted in a variant form of visceral disease in several individuals with confirmed infection with this organism (3, 4). Additional confirmed cases continue to arise at this time. Referred to as viscerotropic leishmaniasis (VTL), these infections differ from classical VL in the variable pathology observed, with several patients lacking both fever and hepatosplenomegaly (4). In addition, serum anti-leishmanial antibody titers are much lower than those observed in patients with classical VL. Diagnosis of classical VL has utilized the elevated antibody response to parasite antigens in tests involving serological reactivity to whole or lysed promastigotes (5, 6) or to recombinant antigens (7). Confirmation is achieved by the isolation of live parasites from spleen, liver, bone marrow, or lymph nodes. Serological reactivity to promastigotes in VTL patients is usually low or absent (4).

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An alternative diagnostic strategy is to identify and apply immunodominant recombinant antigens to increase assay sensitivity and specificity. We report herein the cloning, expression, and evaluation of an immunodominant *L. tropica* antigen¶ capable of both specific antibody detection and elicitation of interferon γ (IFN-γ) production in peripheral blood mononuclear cells (PBMCs) from VTL patients. These results demonstrate the danger of relying on crude immunological assays for the diagnosis of subtle, albeit serious, VTL in Desert Storm patients.

MATERIALS AND METHODS

Parasites. *L. tropica* isolates MHOM/SA/91/WR1063SS, MCAN/SA/91/WR1091SS, MHOM/SA/91/WR1092SS, MHOM/IQ/91/WR1095SS, MHOM/SA/92/WR2044SS; *L. tropica* (Rupert); *L. tropica* (Azad); *Leishmania amazonensis* IFLA/BR/67/PH8; *Leishmania braziliensis* MHOM/BR/75/M2903; *Leishmania chagasi* MHOM/BR/82/BA-2, C1; *L. donovani* MHOM/Et/67/HU3; *Leishmania guyanensis* MHOM/BR/75/M4147; *L. infantum* IPT-1; *L. major* LTM p-2; *L. major* (Friedlander); and *Trypanosoma cruzi* MHOM/CH/00/Tulahuen C2 were used. *Leishmania* promastigotes and *T. cruzi* epimastigotes were cultured in axenic media. *L. major* amastigotes were isolated from infected C.B-17 *scid* mice.

Patient Sera and PBMCs. The VTL patient group included eight culture-positive individuals, seven with confirmed *L. tropica* infection and one with insufficient parasites available for isoenzyme analysis. Four others were culture-negative but either PCR or monoclonal antibody (mAb)-smear-positive. CL patient sera were from M. Grogl (Walter Reed Army Institute of Research, Washington, DC). Normal sera were from the American Red Cross (Portland, OR).

Isolation of Lt-1 and Lt-2. *L. tropica* MHOM/SA/91/WR1063C genomic DNA was isolated and sheared by passage through a 30-gauge needle to 2–6 kb. The library was constructed in Lambda ZapII (Stratagene) by using *Eco*RI adaptors. Expression screening was performed with a pool of preadsorbed patient sera (8).

Expression of Recombinant *L. tropica* Antigens. Induced bacterial pellets were lysed, and recombinant (r) Lt-1, rLt-1r, and rLt-2 were recovered from the inclusion bodies. rLt-1 and rLt-2 were solubilized in 8 M urea and rLt-1r in 4 M urea. The recombinant proteins were purified by ammonium sulfate precipitation and preparative gel separation by SDS/PAGE in

Abbreviations: VTL, viscerotropic leishmaniasis; VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; PBMC, peripheral blood mononuclear cell; IL, interleukin; mAb, monoclonal antibody; TNF-α, tumor necrosis factor α; IFN, interferon; r, recombinant.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U31221).

10% gels. Recombinant proteins were eluted from the gels and dialyzed in phosphate-buffered saline (PBS), the concentration was measured by the Pierce BCA assay, and purity was assessed by SDS/PAGE followed by Coomassie blue staining.

Molecular Analysis of Lt-1 and Lt-2. Exonuclease III digestion was used to create overlapping deletions of the clones (9). Single-strand template was prepared and sequenced with Applied Biosystems automated sequencer model 373A or by Sanger dideoxynucleotide sequencing (10). Both strands of the coding portion of the Lt-1 clone were sequenced.

Genomic DNA was digested with a variety of enzymes, separated by agarose gel electrophoresis, and blotted on Nytran (Scheicher & Schuell). The *L. tropica* inserts were labeled with [^{32}P]dCTP by random oligonucleotide primers (Boehringer Mannheim) and used as probes. Hybridizations involving the Lt-1 or the Lt-2 probes were performed at 65°C, and the hybridizations involving the rLt-1r probe were performed at 60°C in 0.2 M NaH_2PO_4/3.6 M NaCl/0.2 M EDTA overnight and washed to a stringency of 0.075 M NaCl/0.0075 M sodium citrate (0.15 M NaCl/0.0150 M sodium citrate was used with Lt-1r probe), pH 7.0/0.5% SDS at the temperature of hybridization.

Serology. ELISAs were performed as described (6). In competition experiments, sera were preincubated for 30 min with 5 μg of rLt-1r at room temperature. Plates were washed five times with PBS-T (phosphate-buffered saline/0.1% Tween 20). For IgG subclass ELISA tests, plates were coated, blocked, and incubated with sera as described (6). After plates were washed five times with PBS-T, mouse anti-human IgG1, IgG2, IgG3, and IgG4 mAb (Calbiochem) was added (50 μl of a 1:1000 dilution) and incubated 30 min. Plates were washed five times with PBS-T and bound antibodies were detected by goat anti-mouse IgG1-horseradish peroxidase (Zymed; 1:500 dilution) and ABTS substrate. The two-sample *t* test was used in statistical analysis.

Immunoblot Analysis. *Leishmania* parasite lysate, purified rLt-1, and purified rLt-1r were subjected to SDS/PAGE in a 7.5 or 12% gel and transferred to nitrocellulose (15 min at 50V and 1 h at 100V). Filters were blocked with PBS containing 5% (wt/vol) nonfat milk at 4°C overnight, washed three times in PBS-T, and incubated 1 h in human sera (diluted 1:50 in PBS-T) or rabbit sera (diluted 1:250 in PBS-T) on a rocker at room temperature. Filters were washed three times with PBS-T and bound antibody was detected with ^{125}I-labeled protein A at 10^5 cpm/ml followed by autoradiography.

Rabbit antiserum to rLt-1 was raised in an adult New Zealand White rabbit (R & R Rabbitry, Stanwood, WA) by an initial s.c. delivery of 200 μg of rLt-1 in 1 ml of Freund's incomplete adjuvant (Bethesda Research Laboratories) and 100 μg of muramyl dipeptide (Calbiochem), followed by two successive s.c. immunizations of 100 μg of rLt-1 in 1 ml of incomplete Freund's adjuvant at 3-week intervals. A final i.v. boost of 25 μg of rLt-1 was delivered after an additional 4 weeks and serum was collected 2 weeks later.

Proliferative Assays and Cytokine Production. Purified PBMCs were cultured as described (11). Both parasite lysate and rLt-1 were used at 10 μg/ml. Endotoxin levels of all antigens were <5 pg/μg. Supernatants were collected at 72 h for cytokine assays. Capture ELISAs to detect interleukin (IL)-4, IL-10, and IFN-γ were performed as described (11). Capture ELISAs to detect tumor necrosis factor α (TNF-α) used an anti-human TNF-α mAb (Immunex) and a polyclonal rabbit anti-human TNF-α sera (Immunex). Human rTNF-α was used to generate a standard curve with a sensitivity of 40 pg/ml.

Six patient and 6 normal donors were assayed for IL-4 production in response to rLt-1, and 3 patient and 3 normal donors were assayed in response to parasite lysate. Three patient and 5 normal donors were assayed for IL-10 production in response to rLt-1, and 5 patient and 8 normal donors were assayed in response to lysate. Eleven patient and 17 normal donors were assayed for IFN-γ in response to rLt-1, and 9 patient and 13 normal donors were assayed in response to lysate. Five patient and 9 normal donors were assayed for TNF-α production in response to rLt-1, and 2 patient and 4 normal donors were assayed in response to lysate.

Triplicate cultures for proliferation assays were cultured in the presence or absence of antigen for 5 days. Samples were then pulsed with 1 μCi of [^{3}H]thymidine (ICN; 1 Ci = 37 GBq) and incubated 18 h before harvesting. Six patient and six normal donors were assayed for proliferation.

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          V              R        A A          D V    A A   GAAAAAGATGAG 990
  Q A R   Q A L E E A A R L   A E L E    E E A A R L    M H    E  E K D E 330
          S              C        E E          E A    E G

CGGCCGGCGACGTCGAGCTACAGCGAGGAGTGCAAAGGGCGACTGCTATCGAGGGCGCGGCCGGATCCGCGGAGGCCGCTGCCGCGGCCG 1080
 R  P  A  T  S  S  Y  S  E  E  C  K  G  R  L  L  S  R  A  R  P  D  P  R  R  P  L  P  R  P  360

TTCATTGGGATGTCACTGTTGGAGGATGTGGAGAAGAGTATTCTCATTGTGGACGGGCTCTACAGGGATGGGCCGGCGTACCAGACGGGC 1170
 F  I  G  M  S  L  L  E  D  V  E  K  S  I  L  I  V  D  G  L  Y  R  D  G  P  A  Y  Q  T  G  390

ATCCGCCTCGGGGATGTCCTCTTGCGTATCGCGGGGGTTTACGTGGATTCAATAGCGAAGGCGAGGCAGGTGGTCGATGCGCGTTGCCGC 1260
 I  R  L  G  D  V  L  L  R  I  A  G  V  Y  Y  V  D  S  I  A  K  A  R  Q  V  V  D  A  R  C  R  420

TGCGGCTGCGTCGTTCCCGTGACGCTGGCGACGAAGATGAACCAGCAGTACAGCGTGGCTCTGTATATCATGACGGTGGATCCGCAGCAC 1350
 C  G  C  V  V  P  V  T  L  A  T  K  M  N  Q  Q  Y  S  V  A  L  Y  I  M  T  V  D  P  Q  H  450

AACGACAAGCCCTTTTTTTTTGATGTGCACATCCACCACCGCATCGAGAGCTCGCACATGGGGAAGAAGGCGCAGTGGATGGAAGTTCTT 1440
 N  D  K  P  F  F  F  D  V  H  I  H  H  R  I  E  S  S  H  M  G  K  K  A  Q  W  M  E  V  L  480

GAGAGCCCATCCGTATCTTCGGCTGCCACCACCCCTCTCGTGCCGCTCTTGCGTGAGCCGACGCCGCGTAGGGGCTCAGAGCTGCAGTCA 1530
 E  S  P  S  V  S  S  A  A  T  T  P  L  V  P  L  L  R  E  P  T  P  R  R  G  S  E  L  Q  S  510

AGTGCTCGTTCCGCCTTCGTTGCCACGTCTTACTTCTCGAGCGCGCGCAGGTCGGTCAGCTCAGAAAGTGAGCGACCGCGCGGGTCCTCT 1620
 S  A  R  S  A  F  V  A  T  S  Y  F  S  S  A  R  R  S  V  S  S  E  S  E  R  P  R  G  S  S  540

AGCGTGGCTATGGCGGAGGAGGCGATCGCGCTGGCGCCGCAAGGGTATACCCCACCCAACCAAGTGCGCGGCCGTAGTTGACGTCTCTGT 1710
 S  V  A  M  A  E  E  A  I  A  L  A  P  Q  G  Y  T  P  P  N  Q  V  R  G  R  S  *            566

GTGAGTGTGTGTCGCTCCGTCTCCTTCCTTTTTCGTCATGTGTTTTATTCATTTCTTTTTC                              1771
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FIG. 1. Partial DNA sequence of the Lt-1 clone and deduced amino acid sequence of the open reading frame. The DNA sequence of the nonrepeat coding portion of the Lt-1 insert is shown, and the deduced amino acid sequence of both the repeat portion (boldface type) with degeneracies present and the nonrepeat portion. The rLt-1r recombinant protein includes one and one-third of the repeat shown and contains the upper residues shown at the degenerate positions.

RESULTS

Isolation of Recombinant *L. tropica* Clones. Approximately 43,000 recombinant phage were screened by using a pool of preadsorbed patient sera, resulting in the identification of three immunoreactive clones containing inserts of 3.3, 1.4, and 2.7 kb, encoding recombinant antigens of 75, 70, and 110 kDa. These were referred to as Lt-1, Lt-2, and Lt-3, respectively. The recombinant antigens encoded by Lt-1 and Lt-2 were expressed and purified.

Molecular Analysis of *L. tropica* Genes. Sequence analysis of the two *L. tropica* clones was performed. The DNA sequence of the coding portion of the Lt-1 clone includes a repeated sequence at the 5′ portion of the clone containing eight copies of a 99-bp repeat and three copies of a 60-bp repeat unit that is part of the larger 99-bp repeat (data not shown), followed by 800 bp of nonrepeat sequence (Fig. 1). The deduced amino acid sequence of the 99-bp repeat contains limited degeneracies (Fig. 1). The molecular mass of the predicted recombinant protein is 67,060 Da. A database search with the predicted amino acid sequence of the open reading frame yielded no significant homology to previously submitted sequences. Predicted secondary structure of the repeat portion of the clone is entirely α-helical. Sequence analysis of Lt-2 revealed that the 3′ portion of the clone consisted of a mixture of 60- and 99-bp repeats that were identical, excepting occasional degeneracies, to the 60- and 99-bp repeats observed in Lt-1 (data not shown). Collectively, the sequencing data suggest that Lt-1 and Lt-2 are different portions of the same gene, Lt-2 being upstream of Lt-1, with possibly a small overlap. The nested deletion set of Lt-1 formed for sequencing included a deletion clone containing the 5′ portion of the repeat sequence. This clone, referred to as Lt-1r, consists of one and one-third repeats (Fig. 1) and was also expressed as a fusion protein and purified.

Genomic DNA from a number of *Leishmania* species including *L. tropica* were analyzed on Southern blots by using the Lt-1 insert as a probe. Collectively, various digests of *L. tropica* DNA indicated that this gene had a low copy number (Fig. 2). The comparison of hybridization intensities to *Pst* I digests of numerous species yielded a surprising result: the greatest hybridization was observed with members of the *L. donovani* complex including *L. donovani*, *L. chagasi*, and *L. infantum* and lower hybridization was observed with *L. major*, a species that has been considered to be closely related to *L. tropica* (Fig. 2). In addition, weak hybridization was observed with *L. amazonensis*, and none was observed with *L. braziliensis*, *L. guyanensis*, or *T. cruzi*. An overlapping pattern was observed by using the Lt-2 insert as a probe (data not shown), consistent with the premise that these two clones contain sequences near or overlapping one another.

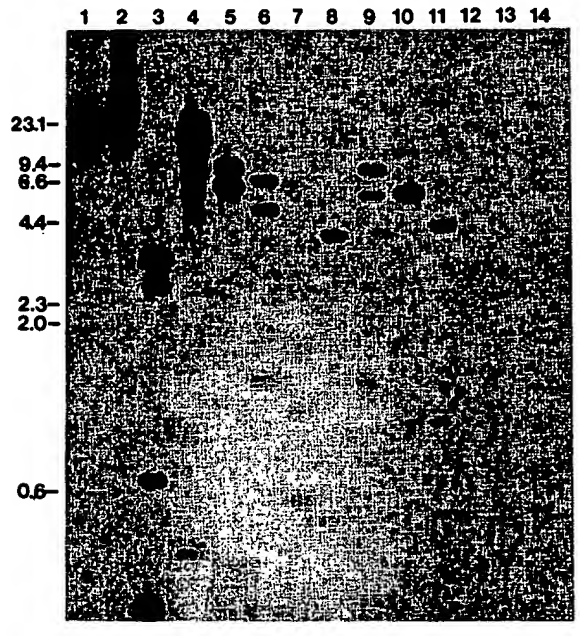

FIG. 2. Southern blot analysis of Lt-1 sequence. Genomic DNA (2.5 μg per lane) of *L. tropica* was digested with *Eco*RI (lane 1), *Xba* I (lane 2), *Xho* I (lane 3), *Bam*HI (lane 4), *Hin*dIII (lane 5), and *Pst* I (lane 6). *L. major* (lane 7), *L. donovani* (lane 8), *L. infantum* (lane 9), *L. chagasi* (lane 10), *L. amazonensis* (lane 11), *L. braziliensis* (lane 12), *L. guyanensis* (lane 13), and *T. cruzi* (lane 14) were digested with *Pst* I. Blots were probed with the Lt-1 sequence. Molecular sizes in kilobases are shown.

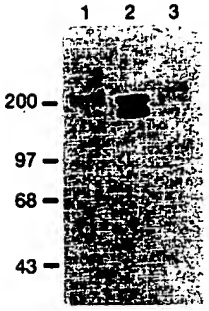

FIG. 3. Reactivity of rabbit anti-rLt-1 sera on *Leishmania* lysates. Bands from SDS/PAGE gels containing 15 μg of *L. major* promastigote lysate (lane 1), 15 μg of *L. major* amastigote lysate (lane 2), and 15 μg of *L. tropica* promastigote lysate (lane 3) were transferred to nitrocellulose and reacted with a 1:250 dilution of rabbit anti-rLt-1 sera. No reactivity was observed by using a similar dilution of the preimmunization sera. Molecular masses in kDa are indicated.

Southern blot analyses of digested genomic DNA from four *L. tropica* parasite strains isolated from VTL patients and three *L. tropica* parasite strains isolated from CL patients (two human and one canine) were performed by probing with the Lt-1r insert. The seven *L. tropica* isolates yielded similar intensities and restriction patterns, with only a single restriction fragment length polymorphism among the isolates (data not shown). These data indicate strong similarity in this region among the *L. tropica* isolates.

To characterize the native *Leishmania* protein that represents the Lt-1 clone, a rabbit antiserum was raised to rLt-1 and used in an immunoblot of *Leishmania* lysates (Fig. 3). Reactivity was observed with a 210-kDa protein in *L. tropica* promastigote lysate (lane 3) and the gene was designated *Lt-210*. Due to the great difficulty of obtaining *L. tropica* amastigotes, we examined the reactivity of rLt-1 antisera with *L. major* promastigote and amastigote lysates, reasoning that the hybridization in Southern blot analysis, albeit weak, indicated the presence of an *Lt-210* homolog in this species. These results (lanes 1 and 2) demonstrated the presence of a cross-

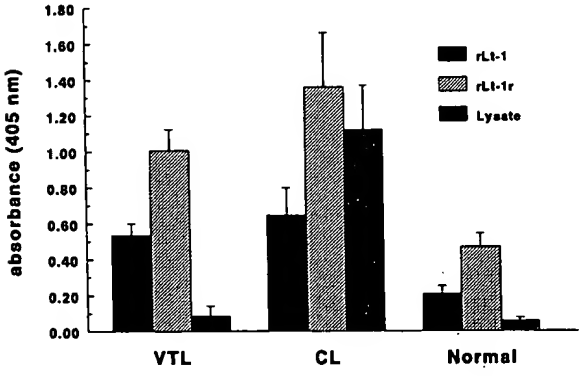

FIG. 4. ELISA evaluation of *L. tropica* infection by using VTL sera or CL patient sera, *L. tropica* lysate (1 μg), and the antigens rLt-1 (50 ng) and rLt-1r (25 ng). A_{405} values (mean + SEM) are shown for VTL patient sera (n = 12), CL patient sera (n = 10), and normal sera (n = 18). Sera were used at a 1:50 dilution.

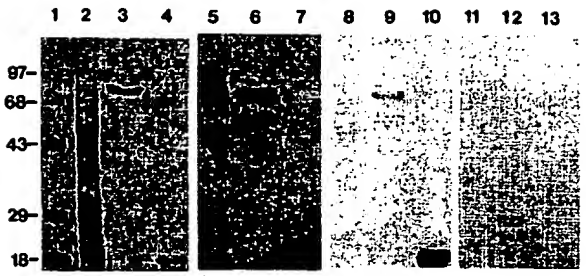

FIG. 5. Immunoblot analysis of *L. tropica* lysate, rLt-1, and rLt-1r. *L. tropica* lysate (15 μg), rLt-1 (2 μg), and rLt-1r (2 μg) on blots were stained with Coomassie blue (lanes 2–4) or reacted with two VTL sera (lanes 5–7 and 8–10) or a pool of normal sera (n = 3, lanes 11–13). Molecular markers are shown in lane 1 and indicated in kDa.

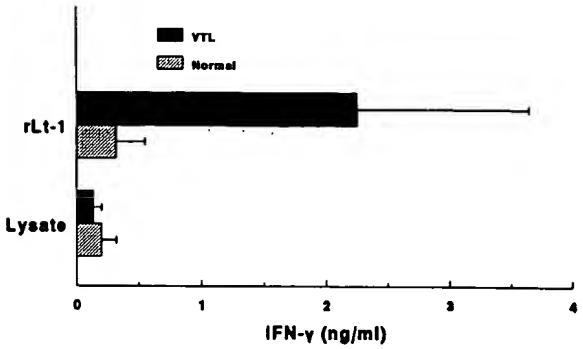

FIG. 7. Stimulation of IFN-γ production by VTL patient PBMCs with rLt-1. Production of IFN-γ (mean + SEM) by VTL patient PBMCs (n = 5) and normal PBMCs (n = 4) in response to incubation with *L. tropica* lysate (10 μg/ml) and the antigen rLt-1 (10 μg/ml) was determined by ELISA analysis of 72-h culture supernatants.

reactive protein of similar size in *L. major* that was most abundant in amastigotes, the form occurring during human infection. An additional amastigote protein of lesser size is also recognized by the rLt-1 antisera and may represent either a related protein or a degradation product of Lt-210.

Patient Sera Reactivity with *L. tropica* Recombinant Antigens. To determine their diagnostic potential, rLt-1 and rLt-1r were evaluated by ELISA with sera from VTL and CL patients and normal sera (Fig. 4). Mean reactivity to rLt-1 was significantly higher in both the CL group (P = 0.002) and the VTL group (P < 0.001) compared to normal sera. A significant increase in reactivity to rLt-1r was also observed in both the CL group (P = 0.001) and the VTL group (P < 0.001) relative to normal sera. In addition, a significant increase in reactivity to rLt-2 was observed in the VTL group (P = 0.036) but not in the CL group (P = 0.279) compared to normal sera (data not shown). Reactivity to *L. tropica* promastigote lysate was significantly increased in the CL group (P < 0.001) but not in the VTL group (P = 0.594), compared to the normal group.

To determine the complexity of epitopes being recognized by patient sera, two VTL sera and one CL serum were preincubated with 5 μg of rLt-1r and tested for reactivity with rLt-1 in an ELISA. In all three cases, the reactivity to rLt-1 was eliminated by preincubation with rLt-1r (data not shown), indicating these individuals were reacting exclusively to epitopes contained within the repeated sequence.

Immunoblot analysis of parasite lysate, rLt-1, and rLt-1r was performed with sera from two VTL patients and a pool of three normal sera (Fig. 5). Increased reactivity to both recombinant antigens was observed in VTL patient sera compared to normal sera. Collectively, the ELISA and immunoblot data indicate that both rLt-1 and rLt-1r have increased specificity compared to promastigote lysate and suggest their utility in a diagnostic assay.

The reactivity of patient sera was further examined by ELISA with IgG-subclass-specific mAbs. Analysis of anti-rLt-1 IgG responses (Fig. 6) demonstrated different subclass distributions in VTL and CL patient sera. A bias toward IgG2 subclass was observed in VTL patient sera, whereas a mixed profile was observed with CL patient sera. In addition, CL IgG responses to *L. tropica* lysate were biased toward IgG1 and IgG3. Normal mean IgG reactivities to rLt-1, when measurable, were biased toward IgG1 and IgG2 (data not shown). Similar results were obtained by substituting rLt-1r for the rLt-1 antigen (data not shown).

Patient PBMC Response to Lt-1. Patient and normal PBMCs were analyzed for proliferation and production of IFN-γ, TNF-α, IL-4, and IL-10, in response to *L. tropica* lysate or purified rLt-1. No significant proliferative responses to either lysate or rLt-1 were observed in patient compared to normal PBMCs. However, rLt-1, but not promastigote lysate, elicited the production of IFN-γ from patient, but not normal, PBMCs (Fig. 7). Collectively, 11 VTL patient and 17 normal PBMC preparations cultured with rLt-1 were assayed, and a significant increase in IFN-γ production was observed with patient PBMCs (P = 0.008). Neither *L. tropica* lysate nor rLt-1 elicited production of detectable levels of IL-4 in patients or normal PBMCs. Similarly, no increase in IL-10 or TNF-α production was observed in patient PBMCs compared to normal PBMCs cultured with lysate or rLt-1.

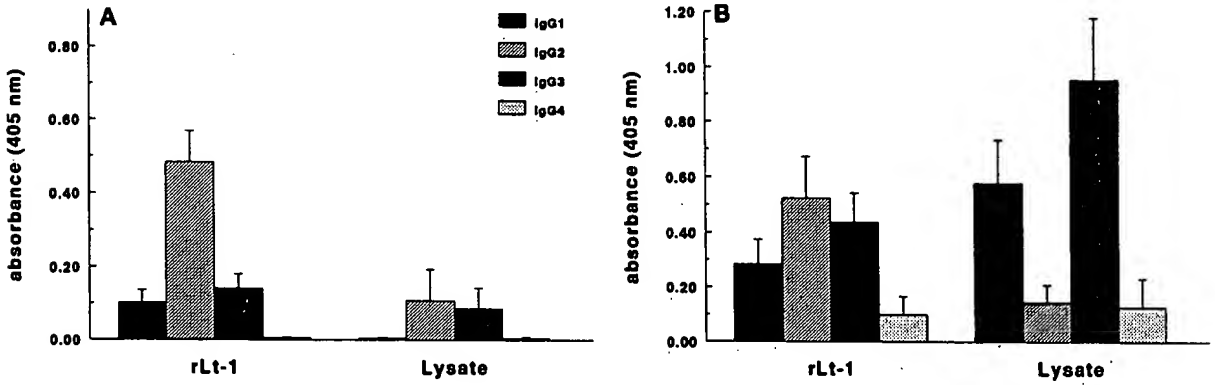

FIG. 6. IgG subclass ELISA evaluation of VTL sera (*A*) and CL sera (*B*) by using *L. tropica* lysate (1 μg) and the antigen rLt-1 (50 ng). A_{405} values (mean + SEM) are shown for VTL patient sera (n = 11) and CL patient sera (n = 10). Sera were used at a 1:50 dilution.

DISCUSSION

We have characterized immune responses of VTL patients to recombinant *L. tropica* antigens, rLt-1 and rLt-1r. These antigens appear to be serologically immunodominant, detecting antibodies in both *L. tropica*-infected VTL and CL patient sera. The molecular characterization of the two *L. tropica* genomic clones analyzed in this study indicated that they shared a domain of repeated sequence. Previous work has demonstrated the presence of serological repeat epitopes within other antigens of *Leishmania* (7) and a related parasite, *T. cruzi* (12, 13). Competition experiments with several patient sera using the Lt-1r gene product demonstrated that the serological epitope of rLt-1 was confined to the repeat portion of the clone.

The data presented herein indicate that the VTL patients are not unresponsive to leishmanial antigens but that the crude lysate most often used in diagnostic assays was unable to detect such responses. In fact, specific serological and cellular responses to rLt-1 indicate that these individuals are responding to at least one immunodominant antigen. To further characterize the serological response to rLt-1 and rLt-1r, the relative contributions of individual IgG subclasses were assessed. It was found the antibody responses to rLt-1 and rLt-1r in VTL patient sera were biased toward IgG2, while CL patient sera showed a mixed IgG profile to the recombinant antigens. CL patient reactivity to *L. tropica* lysate was predominantly of the IgG1 and IgG3 subclasses. These results argue that the contributing factors controlling antibody response in these patients may include properties inherent to the antigen, as demonstrated by the difference between the CL reactivity to rLt-1 and the reactivity to the heterogenous mixture of antigens present in the promastigote lysate. However, the different responses to rLt-1 by VTL and CL sera also suggest that the overall pathology contributes to the serological response to individual antigens.

The observed bias toward IgG2 reactivity observed is unusual since this subclass has been associated with nonprotein T-cell-independent antigens such as bacterial polysaccharides (14, 15). Whether or not the rLt-1 repeat is mimmicking a carbohydrate antigen or is operating through a T-cell-dependent mechanism is unclear. The significance of antigen-specific IgG subclass expression in humans is not known, but some associations have been made between cytokine and isotype profiles. In a T-cell-dependent assay, the addition of IL-4 to human PBMCs induced production of IgE (16, 17). In addition, IL-4 has been implicated in the switching of $sIgM^+$ B cells to IgG4 (18). More recent findings have demonstrated that the addition of IL-10 to $sIgD^+$ $sIgM^+$ B cells activated through CD40 results in secretion of IgG1 and IgG3 (19), while addition of IFN-γ to unstimulated PBMCs results in an increased production of IgG2 (20).

In addition to serological responses, we examined cytokine profiles in PBMCs stimulated with *L. tropica* antigens. Analyses of patient cytokine profiles have been useful for immunological characterization of different clinical forms of leishmaniasis (21, 22). The examination of the cytokine profile from VTL patient and normal PBMCs revealed that rLt-1 elicited significantly higher levels of IFN-γ from VTL patient PBMCs compared to normal PBMCs. These results indicated that rLt-1 was able to elicit the production of a predominantly T helper Th1-associated cytokine, IFN-γ, consistent with a rLt-1-specific IgG2 bias in these individuals, rather than the production of subclasses more closely associated with T helper-Th2 cytokines. Finally, it is interesting to speculate that antigen-specific IFN-γ production may be contributing to the symptoms reported by veterans with unexplained Gulf War-related illnesses, sometimes referred to as Gulf War Syndrome (23).

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EXR. NOTES

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IN-VITRO RESPONSES TO %%%LEISHMANIA%%% ANTIGENS BY LYMPHOCYTES FROM PATIENTS WITH %%%LEISHMANIASIS%%% OR CHAGAS' DISEASE
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ABSTRACT: T cell responses are correlated with recovery from and resistance to %%%leishmaniasis%%%. Antigens of %%%Leishmania%%% chagasi were evaluated by determining their ability to elicit in vitro proliferation and cytokine production in peripheral blood lymphocytes and in T cell lines and clones from patients with histories of %%%leishmaniasis%%% or Chagas' disease. Antigens tested were selected by their reactivity with patients antibodies. Several of the antigens induced proliferative responses in peripheral blood lymphocytes from patients recovered from visceral or cutaneous %%%leishmaniasis%%% or with chronic Chagas' disease. Two purified glycoproteins, 30 and 42 kD, were consistently among the most effective in eliciting high proliferative responses and Il-2 production. Lymphocytes from a recovered visceral %%%leishmaniasis%%% patient were used to produce T cell lines against either the 30- or 42-kD antigen. Each of the lines responded to both of these antigens as well as to crude %%%leishmania%%% %%%lysate%%%. CD4+ T

0006723975 BIOSIS NO.: 198988039090
CHARACTERIZATION OF A PROTEIN FRACTION CONTAINING CYTOCHROMES B AND C-1 FROM MITOCHONDRIA OF %%%LEISHMANIA%%%-TARENTOLAE
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JOURNAL: Experimental Parasitology 68 (4): p443-449 %%%1989%%%
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ABSTRACT: A soluble red band fraction was obtained from %%%Leishmania%%% tarentolae cells by sucrose gradient sedimentation of a Triton X-100
%%%lysate%%%. Spectral

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Vaccination of Balb/c mice against experimental visceral %%%leishmaniasis%%% with the GP36 glycoprotein antigen of %%%Leishmania%%% donovani

De Souza, EP; Bernardo, RR; Palatnik, M; De Sousa, CBP
Instituto de Microbiologia, 'Prof. Paulo de Goes', Universidade Federal do

In Vitro Responses to Leishmania Antigens by Lymphocytes from Patients with Leishmaniasis or Chagas' Disease

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Abstract

T cell responses are correlated with recovery from and resistance to leishmaniasis. Antigens of *Leishmania chagasi* were evaluated by determining their ability to elicit in vitro proliferation and cytokine production in peripheral blood lymphocytes and in T cell lines and clones from patients with histories of leishmaniasis or Chagas' disease. Antigens tested were selected by their reactivity with patient antibodies. Several of the antigens induced proliferative responses in peripheral blood lymphocytes from patients recovered from visceral or cutaneous leishmaniasis or with chronic Chagas' disease. Two purified glycoproteins, 30 and 42 kD, were consistently among the most effective in eliciting high proliferative responses and IL-2 production. Lymphocytes from a recovered visceral leishmaniasis patient were used to produce T cell lines against either the 30- or 42-kD antigen. Each of the lines responded to both of these antigens as well as to crude leishmania lysate. CD4+ T cell clones specific for either or both of these antigens were also isolated from a visceral leishmaniasis patient. In contrast, rabbit antisera produced against these two antigens were not crossreactive. Both antigens were effective in inducing the production of IFN-γ from T cell lines from both leishmaniasis and Chagas' disease patients. These studies demonstrate the potential for defining parasite antigens with broad immunostimulatory capabilities. (*J. Clin. Invest.* 1990. 85:690–696.)
leishmania • antigens • T cells • leishmaniasis

Introduction

The leishmania are a complex of protozoan parasites that cause a spectrum of clinical diseases, including cutaneous, mucosal, and visceral leishmaniasis. They are obligate intracellular parasites of macrophages, which has important immunologic and therapeutic implications. T cell responses are of fundamental importance in leishmaniasis. In visceral leishmaniasis recovery is strongly correlated with a delayed-type hypersensitivity response to leishmania antigens (1–3), as well as with proliferation of peripheral blood lymphocytes (4). Patient's T cells also produce IFN-γ and IL-2 after recovery from acute disease (5). This is of particular interest since IFN-γ has been shown to inhibit the in vitro proliferation of leishmania in macrophages (6). Based on these observations, it appears that evaluation of T cell responses to purified antigens is a logical way to identify molecules of leishmania with diagnostic and immunoprophylactic potential. In the present study we have identified and analyzed isolated antigens of *Leishmania chagasi*, the etiological agent of American visceral leishmaniasis. We have used these antigens to stimulate T cells from patients recovered from disease caused by *L. chagasi* or by the related parasites, *L. braziliensis*, *L. amazonensis*, and *Trypanosoma cruzi*. The parameters used for antigen evaluation were lymphocyte proliferation, IL-2 production by patient's peripheral blood lymphocytes, IFN-γ production by T cell lines, and characterization of T cell clones stimulated by selected antigens.

Methods

Parasite antigen preparation. A clone of *L. chagasi* (MHOM/BR/82/BA-2, C1) was used in all studies (7). Promastigotes were grown to stationary phase (2–3 × 10^7/ml) in RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with L-glutamine, Na pyruvate, MEM essential and nonessential amino acids (Gibco Laboratories), and 10% SeruMax (Sigma Chemical Co., St. Louis, MO). Crude antigen was prepared by sonicating promastigotes in 10 mM Tris, pH 7.5, 2 mM EDTA, 1.6 mM PMSF (Sigma Chemical Co.), and 100 μg/ml leupeptin (Sigma Chemical Co.), followed by centrifugation (5,000 *g*, 20 min). Protein content of the supernatant was determined by the Pierce assay (Pierce Chemical Co., Rockford, IL). Preparative SDS-PAGE was performed by loading 3.2 mg total parasite protein onto a 1.5-mm-thick 12% polyacrylamide gel in sample buffer (50 mM Tris-HCl, 1% SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.01% bromphenol blue) and running by standard procedure. A section of the gel was transferred to nitrocellulose for immunoblotting with patient serum for antigen band identification. Bands of interest were excised and gel slices were diced into 2–3-mm cubes and soaked overnight at 4°C in 2% SDS, 0.4 M NH_4HCO_3, and 0.1% dithiothreitol. The gel pieces and the soaking buffer were then placed into an electro-eluter (Bio-Rad Laboratories, Richmond, CA). Elution occurred for 6–7 h at 10 mA per tube in 0.05 M NH_4HCO_3, 0.1% SDS. The eluted fractions were dialyzed against 0.01 M NH_4HCO_3, 0.02% SDS for 24 h, followed by dialysis against a minimum of 100 vol of PBS, pH 7.4, for 3–5 d with two buffer changes per 24 h. All dialysis was done at 4°C. Eluted samples were assayed for protein content (Pierce assay) and checked for purity on SDS-PAGE mini-gels with silver staining (Bio-Rad Laboratories). High molecular weight protein standards (Bethesda Research Laboratories, Bethesda, MD) were used.

Proteolysis treatments. Some of the eluted fractions were treated with cyanogen bromide (CnBr; Eastman Kodak Co., Rochester, NY), trypsin (Boehringer Mannheim Biochemicals, Indianapolis, IN), and endolys C (Wako Chemicals USA, Dallas, TX). Proteolysis reactions occurred under the following conditions: CnBr; 2 parts CnBr:1 part protein (wt/wt), incubated in 70% formic acid, 30% water at room temperature for up to 16 h. Trypsin; 25 parts protein dissolved in 50

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mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA 10 mM Pipes buffer, pH 7.8; 1 part enzyme (wt/wt), incubated at room temperature for 16 h. Endolys C; 400 parts protein dissolved in 50 mM Tris, pH 8.0:1 part enzyme (wt/wt), incubated at 30°C for 16 h.

Immunoblotting. The electrophoresed parasites or fractions were transferred to nitrocellulose and the sheets were blocked and reacted with sera as described (7). Biotinylated goat anti-human IgG, A, and M (Zymed Laboratories, So. San Francisco, CA) was applied in a dilution of 1:8,000 in PBS-T and incubated for 30 min, followed by two washes in PBS-T and a final wash in PBS. Avidin-alkaline phosphatase (Zymed Laboratories) was then applied, diluted 1:1,500 in PBS, and incubated for 30 min. After three washes substrate was added consisting of nitro blue tetrazolium (330 μg/ml; Sigma Chemical Co.) and 5-bromo-4 chloro-3 indolyl phosphate (165 μg/ml; Sigma Chemical Co.) in 100 mM Tris (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$.

Lectin blotting. Nitrocellulose strips were prepared as above, except that blocking was performed in BSA (1% in PBS, pH 7.0). After rinsing, the strips were incubated in the following biotinylated lectins (Sigma Chemical Co.): *Lens culinaris* (lentil lectin), *Arachis hypogaea* (peanut lectin), *Phaseolus vulgaris* (PHA), *Canavalia ensiformis* (Con A), and *Triticum vulgaris* (wheat germ) at 1:400 in PBS-T at room temperature for 30 min. After this incubation the strips were washed and incubated in avidin alkaline phosphatase and developed as above.

Antiserum production. Rabbits (adult New Zealand White; R & R Rabbitry, Stanwood, WA) were immunized with eluted protein purified to a single band on an SDS gel for the production of polyclonal antisera. The immunization procedure consisted of two subcutaneous injections of 100 μg protein, together with 500 ng recombinant human IL-1-β in Freund's incomplete adjuvant, given 30 d apart. Intravenous injections of 40 μg protein were given 30 and 60 d after the second subcutaneous injection, and sera were collected 6 d after the final injection.

Biosynthetic labeling and radioimmunoprecipitation. Promastigote lysate was prepared as above, except that the parasites were first incubated (4×10^7/ml in medium containing 20 μCi/ml [^{35}S]methionine (New England Nuclear, Boston, MA) for 14 h with shaking. 100 μl of a 50% suspension of protein A–Sepharose beads (Zymed) were equilibrated in 50 mM $NaBO_3$, pH 8.2, then combined with 25 μl rabbit serum and mixed for 30 min at room temperature. The beads were pelleted and suspended in cross-linking buffer (200 mM triethanolamine, pH 8.2, with 6.1 mg/ml dimethyl pimelimidate · 2 HCl (Pierce Chemical Co.), and mixed for 1 h at room temperature. The beads were then washed twice with cross-linking buffer and blocked with 100 mM ethanolamine, pH 8.2, for 10 min. Beads with bound antibody were equilibrated in PBS, pH 7.2, for use and storage.

For immunoprecipitation, 20 μl of a 50% suspension of the conjugated beads was mixed with 50 μl parasite lysate at 4°C for 2 h. The beads were washed twice with PBS (pH 7.2) containing 0.02% NaN_3, twice with mixed detergent buffer (0.05% NP-40, 0.01% SDS, 0.3 M NaCl, 10 mM Tris HCl, pH 8.6), and twice with PBS-azide. The beads were subjected to standard SDS-PAGE and autoradiography.

Leukocytes. PBL donors were as follows: (*a*) Visceral leishmaniasis; cells were taken from individuals living in an area endemic for visceral leishmaniasis with a history of acute disease due to *L. chagasi* infections 6 mo to 4 yr previously, confirmed by isolation and identification of the parasite. (*b*) Mucosal leishmaniasis; cells were from patients with active mucosal disease or with recently (< 1 yr) cured mucosal infections. (*c*) Cutaneous leishmaniasis; cells were collected from individuals with a recent history (6 mo to 2 yr) of simple cutaneous leishmaniasis, manifested as a single lesion. Patients in groups 2 and 3 were from areas endemic for *L. braziliensis* and had parasite-confirmed infections. (*d*) Chagas' disease; these patients had chronic Chagas' disease and were clinically, serologically, and parasitologically positive for *T. cruzi* infection. (*e*) Healthy controls; these cells were obtained from individuals living in areas endemic for leishmaniasis and Chagas' disease but without a history of these infections.

T cell lines and clones. T cell lines were generated from patients recovered from acute visceral leishmaniasis or cutaneous leishmaniasis and a patient with chronic *T. cruzi* infection. PBL were separated on lymphocyte separation medium (Organon Teknika Corp., Durham, NC) and frozen in liquid nitrogen in aliquots of 2×10^7 in 1 ml. T cell cultivation medium consisted of RPMI 1640 (Gibco Laboratories) supplemented with L-glutamine, Na-pyruvate, 2-mercaptoethanol, and 10% screened human A^+ or AB^+ serum (complete medium). T cell lines were also generated using purified antigens instead of crude lysate.

To generate clones, PBL were cultured for 5 d with crude leishmania (leishmaniasis patients) or *T. cruzi* (Chagas' patients) lysate (10^6 cells per ml, 2 ml per well in a 24-well tray, incubated 5 d with 5 μg parasite protein/ml). T cell blasts, separated in lymphocyte separation medium, were cloned and subcloned by limiting dilution at 0.3 cells/well in 96-well, round-bottom plates (No. 25805; Corning Glass Works, Corning, NY). Clonality was determined according to frequency of positives at limiting dilutions by established methods (8, 9). Clones were maintained by biweekly feedings with human recombinant IL-4 (100 ng/ml), and by stimulation every 10–14 d with irradiated (3,500 rad) autologous whole PBL and leishmania lysate (5 μg/ml) with human recombinant IL-2 (10 ng/ml).

Proliferative assays and cytokine production. 7–10 d after antigen stimulation, 10^4 cloned T cells were cultured with 10^5 irradiated autologous feeder PBL in 200 μl complete medium in triplicate wells of 96-well plates (No. 3595; Costar, Cambridge, MA) with or without 0.5–1 μg antigen protein/well. After 3 d incubation the cells were pulsed overnight with [^{3}H]thymidine (0.5 μCi/well). Stimulation index is counts per minute of culture with antigen/counts per minute of culture without antigen. For IFN-γ and IL-2 production, supernatants were collected after 24–96 h incubation and assayed for IFN-γ by the WISH cell bioassay (10), or for IL-2 by the CTLL bioassay (11).

Results

PBL proliferative responses to parasite lysate or to partially purified antigens. Major antigenic bands of the SDS-PAGE–separated *L. chagasi* were identified using a serum pool from patients recovered from visceral leishmaniasis (Fig. 1). Sera from normal individuals did not recognize leishmania antigens, as we have previously shown (7). In initial studies, bands of M_r 116, 78, 72, 70, 65, 42, 40, 30, and 16 kD were chosen for use in in vitro proliferation assays. These bands were chosen based on their reactivity with sera from visceral leishmaniasis patients and with sera from Chagas' patients (Fig. 1). Several bands, for example the 42- and 30-kD antigens, were recognized by Chagas' patient sera, as well as by leishmaniasis sera, while others, such as the 65-kD antigen, were recognized by leishmaniasis but not Chagas' patient sera. Coomassie

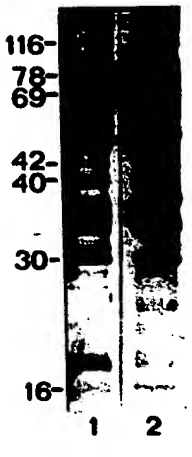

Figure 1. Immunoblot of whole *L. chagasi* lysate (10 μg protein/lane) separated by SDS-PAGE and reacted with pooled sera from five individuals with cured visceral leishmaniasis diluted 1:300 (lane *1*) or from five patients with chronic *T. cruzi* infection diluted 1:100 (lane *2*).

blue-stained bands corresponding to the desired antigenic regions were excised and eluted from the gel and dialyzed to remove SDS. The purity of the fractions was ascertained on polyacrylamide gels before use in cell stimulation assays. All bands retained their antigenicity, as indicated by their ability to bind antibody from patients with cured visceral leishmaniasis on immunoblots (not shown). All proliferative assays were performed with antigens purified to a single SDS-PAGE band (Fig. 2).

The results from testing normal or patient cells for their ability to proliferate in response to crude homogenate or to representative partially purified leishmania antigens are shown in Table I. Several of the antigens were effective in stimulating PBL from visceral, mucosal, and cutaneous leishmaniasis patients, while none of the antigens tested stimulated significant proliferation in cells from normal individuals. Antigens of 30, 40, 42, and 70 kD were effective (> 10-fold proliferation above background) in stimulating significant proliferative responses in PBL cultures from leishmaniasis patients (Table I). However, two bands, 30 and 42 kD, also elicited responses in cultures of PBL from Chagas' patients. These two bands were the only partially purified antigens tested that consistently elicited high responses from all three patient groups, and they were therefore chosen for further study. In subsequent analyses, other antigens were assayed for their ability to elicit proliferation in patient PBL, but none were consistently more effective than those represented in Table I.

Cytokine production by PBL and T cell lines after exposure to leishmania antigens. The production of IL-2 by patient PBL (Table II) was found to correlate directly with proliferation responses. In these experiments lysate of leishmania or *T. cruzi* were compared with selected antigen bands for their ability to induce IL-2 in patient PBL. Initial experiments with whole leishmania lysate showed that maximal IL-2 production occurred in cells from leishmaniasis patients 48 h after antigen stimulation as compared with 24, 72, and 96 h (not shown). Therefore, cultures were established as in proliferation assays, and supernatants were collected for IL-2 determination 48 h after adding antigen. In general, antigens of 70, 42, and 30 kD compared favorably with whole leishmania lysate in stimulating IL-2 production from all three groups of leishmaniasis patients as well as in Chagas' disease patients. None of the antigens tested elicited IL-2 production in PBL from normal individuals.

Figure 2. Analysis of excised bands from preparative SDS-PAGE gels of *L. chagasi* lysate. 5 μg *L. chagasi* lysate (lane *2*) or 1 μg protein of eluted bands of M_r 69, 42, 40, and 30 kD (lanes *3–6*) were analyzed on SDS-PAGE with silver strain. Lane *1* is high molecular weight standards.

T cell lines generated from patients with cured visceral or cutaneous leishmaniasis or with chronic Chagas' disease were tested for their ability to produce IFN-γ in response to the 30- and 42-kD antigens. The results are shown in Table III. Cell lines from the two groups of leishmaniasis patients (visceral and cutaneous) produced significant levels of IFN-γ in response to crude *L. chagasi* lysate, as we have previously reported for patient PBL (5). The production of IFN-γ was maximum at 72 h (data not shown). In addition, both the 30- and 42-kD antigens elicited significant IFN-γ production by T cell lines from both patient groups. Of particular interest was the ability of these antigens, as well as crude lysate, to elicit IFN-γ production in T cell lines from Chagas' patients.

T cell lines were also generated using purified 30- and 42-kD antigens by incubating PBL from a patient recovered from visceral leishmaniasis with gel-eluted protein. Lines were selected and restimulated using only the 30- or 42-kD antigens and tested after 30 d in culture for responsiveness to crude lysate or to partially purified antigens. The results are shown in Table IV. Both of the T cell lines responded to crude leishmania lysate. In addition, the T cell line generated against the 30-kD antigen responded to both the 30- and 42-kD antigens. The same was true for the line generated against the 42-kD antigen. This proliferation could not be explained by the survival of nonselected T cells from the PBL; cultures of the same PBL without added antigen (30- or 42-kD proteins) did not survive past day 10 of culture. These results suggest that T cell epitopes may be shared by the 30- and 42-kD antigens.

To further characterize T cells that responded to the 30- and 42-kD antigens, T cell clones that were specific for these antigens were identified. 148 T cell clones were generated from a patient recovered from acute visceral leishmaniasis. Several of these clones that responded to crude antigen with proliferation > 30-fold above background were also tested for proliferative responses to the 42- and 30-kD fractions. Of 39 clones tested for proliferation to the 30-kD antigen, 15 responded with a stimulation index of 10-fold or higher (Table V). By the same criterion, 11 of 22 clones tested responded to the 42-kD *L. chagasi* antigen. The majority of the clones responded to both antigens, but two responded to the 30- and not the 42-kD antigen, while one responded to the 42- and not the 30-kD antigen. All of these clones were CD4+.

Partial characterization of the 42- and 30-kD fractions. Purity of the eluted fractions was obtained to the level of a single band on an SDS-PAGE gel (Fig. 2). The antigens were treated with different proteolytic agents, including CnBr, trypsin, and endolys C. Both antigens were digested by each of these treatments (Figs. 3 and 4), demonstrating their protein composition. In addition, each of the fractions bound all of the lectins used, as determined by incubation with biotinylated lectin followed by avidin-conjugated phosphatase. Binding of lentil lectin is illustrated in Fig. 5. These results indicated that the 42- and 30-kD fractions appeared to be glycosylated.

Rabbit antisera raised against these two proteins demonstrated that they have unique antigen determinants. Sera from rabbits immunized with either antigen were immunoblotted on or used to immunoprecipitate leishmania lysates (Fig. 6, *a* and *b*). Each of the sera bound to the respective antigens, and neither bound to both antigens. This was true whether the sera were reacted with denatured (Fig. 6 *a*) or nondenatured (Fig. 6 *b*) leishmania lysate, illustrating that the 30-kD antigen is not likely to be a breakdown product of the 42-kD antigen. Fur-

Table I. Proliferation of PBL from Patients with Past or Active Leishmaniasis or Chagas' Disease in Response to Antigens of L. chagasi

Antigen (1 μg protein/well)	[³H]Thymidine uptake				
		Leishmaniasis			
	Normal (n = 10)	Visceral (n = 8)	Mucosal (n = 3)	Cutaneous (n = 4)	Chagas' disease (n = 8)
M_r	mean cpm × 10^{-3}±SD				
116	0.3 (0.2)	1.7 (1.4)	4.8 (3.2)	0.9 (1.1)	2.9 (2.0)
78	0.3 (0.1)	4.2 (0.9)	3.9 (2.0)	7.1 (6.5)	3.1 (2.1)
70	0.6 (0.2)	4.1 (2.4)	18.1 (9.5)*	12.8 (4.2)	6.5 (2.9)
65	0.2 (0.2)	7.9 (4.1)	20.2 (8.3)*	28.0 (5.5)	7.7 (5.2)
42	0.3 (0.1)	13.5 (3.6)*	37.6 (12.4)*	31.1 (8.0)	18.2 (6.4)*
40	0.4 (0.2)	6.5 (3.8)	14.2 (10.1)	15.2 (6.9)	9.1 (6.9)
30	0.4 (0.3)	12.7 (4.8)*	45.8 (12.5)*	46.1 (10.0)	21.5 (6.3)*
16	0.4 (0.5)	0.9 (1.2)	9.6 (3.5)*	7.7 (3.2)	5.5 (4.0)
None (medium only)	0.2 (0.3)	0.5 (0.7)	1.2 (1.6)	1.4 (1.1)	3.1 (1.1)
Leishmania lysate	0.4 (0.3)	19.2 (6.4)*	72.1 (12.8)*	58.5 (9.3)	16.7 (5.1)*
T. cruzi lysate	0.2 (0.3)	ND	ND	ND	51.8 (7.9)*

10^5 PBL were incubated for 5 d in the presence of antigen after a 12-h pulse with [³H]thymidine. * Significant ($P < 0.05$) proliferation above background (medium only wells).

ther, these data suggest the presence of unique antigenic determinants on glycoprotein (gp)[1] 42 and gp 30. The rabbit antiserum raised against gp 30 also precipitated a band of ~ 55–60 kD. We are in the process of characterizing this molecule(s).

Discussion

PBL proliferative response to leishmania antigens has been used as a prognostic indicator for recovery from leishmaniasis. Proliferation of PBL in response to leishmania antigen is negative in patients with acute visceral leishmaniasis but occurs after successful therapy (4). In addition to proliferative responses, we have shown a correlation between the ability of patient PBL to produce IL-2 and IFN-γ in response to leishmania antigens after recovery from acute infection (5). We have therefore chosen proliferation and cytokine production to evaluate human lymphocyte responses to selected leishmania antigens.

The approach used in this study to identify antigens of leishmania that may be important in stimulating leishmania-specific T cell activity was to elute them from gels and use them in soluble form for lymphocyte assays. This can have advantages over the Western blot approach of antigen analysis, in which proteins transferred to nitrocellulose are used for in vitro lymphocyte assays. The use of antigens transferred to nitrocellulose makes it difficult to ensure either quantitatively or qualitatively that the same antigenic stimulus is consistently applied to each well. In contrast, when eluted soluble protein is used, it is possible to standardize a single lot of antigen for repeated testing.

Several antigens of *L. chagasi* stimulated PBL proliferative responses in the homologous patient group (persons with previous acute visceral leishmaniasis due to *L. chagasi*). Some of the antigens also elicited consistently high responses in PBL from cutaneous or mucosal leishmaniasis and in PBL from Chagas' disease patients. Cross-reactivity between antigens of different *Leishmania* species and between antigens of leishmania and *T. cruzi* occurs serologically (7, 12), and also in the PBL proliferation assay (13). Little has been done thus far to characterize the molecules that the parasites in these groups may have in common. Of the several proteins and glycoproteins shared by the leishmania that cause human disease, a 63–65-kD surface antigen appears to be highly conserved among these species and subspecies as judged by serological cross-reactivity (7, 14, 15), and by DNA sequence analysis (our unpublished data). This antigen from *L. chagasi* appeared at 63–65 kD. This antigen is not recognized serologically by individuals with *T. cruzi* infections (7). However, it is interesting to note that this antigen was among the most effective in stimulating T cells from both leishmaniasis and Chagas' patients. In

Table II. Production of IL-2 by PBL from Patients with Leishmaniasis or Chagas' Disease in Response to Antigens of L. chagasi

Antigen	Units IL-2 produced*				
		Leishmaniasis patients			Chagas' disease patients
	Normal	Visceral	Mucosal	Cutaneous	
L. chagasi lysate	0	29±5	24±11	32±0	23±12
T. cruzi lysate	0	ND	ND	ND	48±23
116	0	0	0	0	0
65	0	27±5	18±22	16±0	8±4
42	0	27±5	35±12	27±5	24±8
30	0	37±9	36±26	29±17	31±18

* Units of IL-2 were determined in cultures identical to those in Table I 48 h after antigen stimulation.

1. *Abbreviations used in this paper:* gp, glycoprotein.

Table III. In Vitro IFN-γ Production by T Cells Stimulated with L. chagasi 30- and 42-kD Antigens

Patient group*	IFN-γ‡				
	Medium	*L. chagasi* lysate	Gp 30	Gp 42	*T. cruzi* lysate
		U/ml			
Cured visceral leishmaniasis§	0	32±0	21±4	21±9	ND
Cutaneous leishmaniasis§	0	36±8	16±0	32±0	ND
Chagas' disease‖	0	16±0	16±0	16±0	8±0

* No IFN-γ was observed by cells from normal individuals stimulated with any of the antigens.
‡ IFN production was determined in unconcentrated culture supernatants from 5×10^4 T cells and 10^5 irradiated autologous feeder cells cultured from 1 μg antigen protein for 72 h.

Table V. Proliferation of L. chagasi-specific T Cell Clones to Gp 30 or Gp 42

Antigen	No. clones positive*/ No. tested	[³H]Thymidine uptake	Stimulation index‡
		mean cpm $\times 10^{-3} \pm SD$	*mean±SD*
Gp 30	13/39	9.4±8.6	23.5±15.0
Gp 42	11/22	14.0±12.5	42.0±33.1
L.d. chagasi lysate	39/39	32.9±17.2	49.8±34.0

Response of 10^4 T cells cultured with 10^5 irradiated autologous PBL and 1 μg protein of the indicated antigen, determined after 3 d of culture.
* ≥10-fold stimulation index.
‡ Mean value of responding clones only.

this regard, purified gp 63 from *L. major* was suggested to have some effectiveness in the induction of protective T cell responses in mice (16).

Serological cross-reactivity was useful in selecting leishmania antigens for partial purification. Both gp 42 and gp 30 were identified initially on the basis of their reactivity with sera from patients with different forms of leishmaniasis and with sera from Chagas' disease patients. Although antigenic determinants recognized by B cells may differ from those recognized by T cells, as illustrated in studies on influenza virus (17, 18), selection of a protein for studies of T cell responses was facilitated in this study by observations on antibody reactivity.

In the present study we have begun to characterize selected antigens of leishmania that are able to stimulate T cells from individuals with past leishmania infections or with chronic *T. cruzi* infections. At least two antigens, gp 30 and gp 42, elicited consistently high proliferative responses in all three patient groups to levels roughly equivalent to those obtained using crude leishmania extract. Many T cell clones generated from a patient with cured visceral leishmaniasis responded to both of these antigens, and T cell lines generated against each of the antigens responded to both antigens. These findings, together with the observation that both antigens were among the most effective in stimulating PBL proliferation and IL-2 production suggests that these antigens may have one or more common epitopes. That they may have unique epitopes as well was indicated by the finding of several T cell clones, isolated after subcloning, which respond to one but not both of the antigens, and by the production of non–cross-reactive rabbit antisera. Gp 30 and gp 42 may be major antigens for T cells, as indicated by the relatively high percentage of the clones analyzed that were specific for these antigens (13 of 39 tested and 11 of 22 tested, respectively). In addition, both gp 30 and gp 42 were effective in eliciting the production of IFN-γ from T cell lines from patients with either leishmaniasis or Chagas' disease. This may be an important consideration for selecting antigens as candidates for immunoprophylaxis because of the demonstrated importance of IFN-γ in mediating intracellular destruction of leishmania (6). Additionally, it is of interest that all of the gp 30– and gp 42–specific T cell clones isolated were CD4+.

Table IV. Proliferation of Selected T Cell Lines to the 30- and 42-kD Antigens

Antigen	[³H]Thymidine uptake			
	T cell lines			
	gp 30	Stimulation index	gp 42	Stimulation index
	mean cpm $\times 10^3 \pm SD$			
L. chagasi lysate (2 μg protein/well)	17.6±3.1	11.8	15.4±1.9	9.4
Gp 42 (0.5 μg protein/well)	7.2±2.6	4.9	8.6±1.8	5.2
Gp 30 (0.5 μg protein/well)	9.2±2.8	6.2	7.9±3.6	4.8
None	1.4±2.8		1.6±0.5	

* T cell lines were selected by stimulation of PBL from a patient recovered from visceral leishmaniasis with the 30- or 42-kD antigens eluted from gels (Fig. 1).

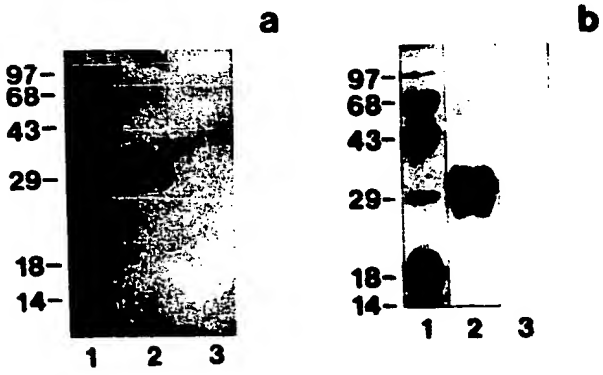

Figure 3. The 30-kD *L. chagasi* glycoprotein is susceptible to proteolytic treatment. 1 μg protein of the eluted 30-kD band (*a* and *b*, lane *2*) was treated with CNBr (*a*, lane *3*) or trypsin (*b*, lane *3*) as described. *a* and *b*, lane *1*, high molecular weight standards (Bethesda Research Laboratories).

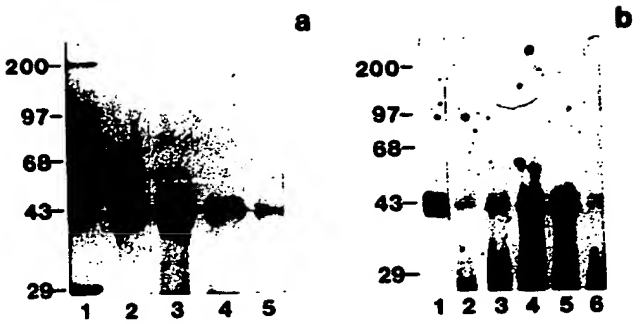

Figure 4. The 42-kD glycoprotein is susceptible to proteolytic treatment. *a*, 1 μg protein of the eluted 42-kD band (lane *2*) was incubated with CNBr (2 parts CNBr:1 part protein) for 2.5 h (lane *3*), 6 h (lane *4*), or 16 h (lane *5*). Lane *1*, high molecular weight standards (Bethesda Research Laboratories). *b*, 1 μg protein of the eluted 42-kD band (lane *1*) was incubated with endolys C at 400 parts protein: 1 part enzyme wt/wt (lane *2*), 800 parts protein: 1 part enzyme (lane *3*), and 3,200 parts protein: 1 part enzyme (lane *4*) for 16 h. In lane *5*, 1 μg of the eluted 42-kD band was incubated with trypsin, 100 parts protein: 1 part enzyme, wt/wt (lane *5*), or 25 parts protein: 1 part enzyme (lane *6*) for 16 h.

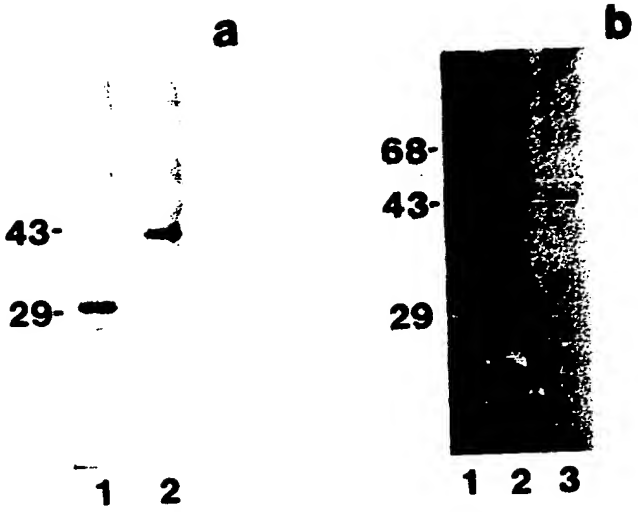

Figure 6. a, Immunoblot of *L. chagasi* lysate using rabbit antisera produced against the 30-kD (lane *1*) and the 42-kD (lane *2*) antigens. 5 μg lysate protein/lane. *b*, Radioimmunoprecipitation of *L. chagasi* lysate using the same sera (lanes *1* [42 kD] and *2* [30 kD]) and normal rabbit serum (lane *3*).

The finding of leishmania antigens that stimulate proliferation and cytokine production by lymphocytes from Chagas' disease patients may be of particular significance. Experimentally, mice have been immunized against acute *T. cruzi* infection by prior inoculation of *L. braziliensis* (19) or *L. chagasi* (Reed, S. G., unpublished results). These observations, together with the in vitro findings in this report, suggest the potential of using leishmania antigens to immunize against *T. cruzi*. The finding that leishmania antigens induced IFN-γ production by Chagas' patient T cells is relevant with regard to mechanisms of immune protection against acute *T. cruzi* infection. It has been shown that IFN-γ can mediate resistance in experimental *T. cruzi* infections (20). Finally, because of the possibility that certain pathological manifestations of Chagas' disease are due to autoimmune responses induced by *T. cruzi* antigens (reviewed in reference 21), the potential of immunizing against this infection with antigens other than those of *T. cruzi* may be of particular interest.

The possibility that the leishmania antigen–induced proliferation and cytokine production of PBL and T cell lines from Chagas' disease patients was due to multiple infections and not cross-reactivity is extremely slight. All of the Chagas' patients tested were from areas that are not endemic for leishmaniasis, and none had clinical histories, signs, or symptoms of any form of leishmaniasis. We have shown how the possibility of dual infection may be ruled out in a previous report (3).

In conclusion, we have begun to analyze antigens of leishmania that stimulate T lymphocyte responses in patients with treated leishmaniasis. The emphasis in this study was to characterize antigens that are capable of stimulating T cell responses from patients with infections caused by related parasites with the goal of identifying molecules having the potential to induce protective immune responses against more than one organism.

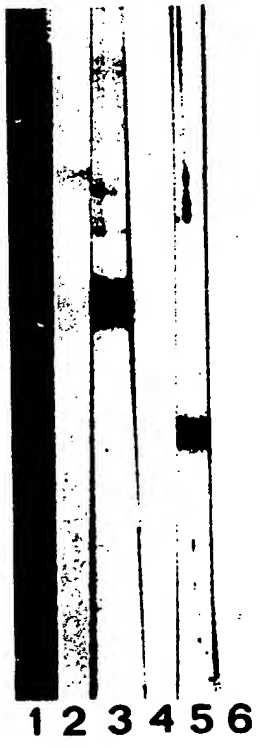

Figure 5. Treatment of eluted *L. chagasi* bands with lectin. Lanes *1* and *2*, whole *L. chagasi* lysate; lanes *3* and *4*, 42-kD eluted protein; lanes *5* and *6*, 30-kD eluted protein. Proteins were transferred to nitrocellulose and treated with biotinylated *Lens culinaris* (lanes *1*, *3*, and *5*) followed by alkaline phosphatase avidin, or with alkaline phosphatase avidin only (lanes *2*, *4*, and *6*), before addition of substrate.

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EXR NOTES

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Author(s): Mukhtar MM (REPRINT) ; Sharief AH; ElSaffi SH; Harith AE; Higazzi TB; Adam AM; Abdalla HS
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Author(s): Streit JA; Recker T
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Author(s): TADOKORO CE; MACEDO MS; ABRAHAMSOHN IA
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Abstract: The adjuvant activity of saponin for T-cell responses was evaluated and compared with that of complete Freund's adjuvant (CFA) in two antigen systems: a %%%lysate%%% of the protozoa Trypanosoma cruzi and ovalbumin (OA). Strong delayed-type hypersensitivity and T-cell proliferate responses, comparable with those stimulated by CFA, were observed for both antigens following immunization with saponin as

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Saponin adjuvant primes for a dominant interleukin-10 production to ovalbumin and to *Trypanosoma cruzi* antigen

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SUMMARY

The adjuvant activity of saponin for T-cell responses was evaluated and compared with that of complete Freund's adjuvant (CFA) in two antigen systems: a lysate of the protozoa *Trypanosoma cruzi* and ovalbumin (OA). Strong delayed-type hypersensitivity and T-cell proliferate responses, comparable with those stimulated by CFA, were observed for both antigens following immunization with saponin as adjuvant. Upon *in vitro* secondary antigen stimulation, high interleukin-10 (IL-10) and low interferon-γ (IFN-γ) levels were observed in lymph node (LN) cell cultures from saponin-immunized mice in contrast with the high IFN-γ and decreased IL-10 production by LN cells from CFA-immunized mice. Production of IL-10 and IFN-γ in these conditions was CD4-activation dependent. Concanavalin A (Con A)-stimulated interleukin-4 (IL-4) production was higher in saponin-immunized mice than in CFA-immunized mice. IL-10 produced by LN cells from saponin-immunized mice suppressed IFN-γ production and Con A-induced proliferation. Taken together, these data are consistent with *in vivo* stimulation of both T-helper (Th)1 and Th2-type cells by immunization with saponin; *in vitro* a Th2-type cytokine response with high IL-10 production predominates, indicating preferential priming towards a Th2-type response. Immunization with CFA induced a Th1-type cytokine response. To our knowledge, this is the first report in which an adjuvant is shown to prime for a dominant IL-10 production.

INTRODUCTION

Saponin is a mixture of triterpene glycosides extracted from the bark of the tree *Quillaia saponaria*. It has been widely used as an adjuvant for foot-and-mouth disease vaccines[1] and for experimental immunization against diseases caused by protozoa.[2,3] More recently various Quil A saponin derivatives with adjuvant activity have been characterized.[4] The antibody isotypes stimulated in the mouse by saponins as adjuvants are immunoglobulin (Ig)G1[5–8] associated with IgG2b[6,7] and/or with IgG2a[4,5,7,8] depending on the antigen and protocols used for immunization. The pattern of induced antibody isotypes allied to the enhanced secretion of interferon-γ (IFN-γ) and interleukin-2 (IL-2)[6] or of IFN-γ and interleukin-5 (IL-5)[5] is considered consistent with the stimulation of both T helper (Th)1 and Th2-type cytokine responses by immunization protocols that use saponin as adjuvant. Among a panel of different adjuvants that included muramyl dipeptide, alum, Freund's incomplete adjuvant, *Corynebacterium parvum* and squalane, saponin was the only one to induce delayed-type hypersensitivity (DTH) to a purified glycoprotein from the protozoa *Trypanosoma cruzi* and partial protective immunity to a challenge with live parasites.[7] Complete Freund's adjuvant (CFA) stimulates strong antibody and cellular immune responses and is considered to preferentially[9,10] stimulate Th1-type responses. However, the intense inflammatory reaction at the site of inoculation and the additional immunization to mycobacteria antigens can constitute drawbacks to its use in protocols of protective immunization.

This paper reports a comparison of cell-mediated immune responses stimulated by saponin or by CFA as adjuvants in two antigen systems: a lysate of the protozoa *T. cruzi* and ovalbumin (OA) as a defined protein antigen. We analysed *in vivo* expression of cutaneous hypersensitivity, lymphoproliferative and cytokine responses to antigen and mitogen following *in vitro* stimulation. Both adjuvants induced DTH. The cytokine profile upon *in vitro* stimulation with antigen showed high levels of interleukin-10 (IL-10) production in saponin-immunized mice whereas CFA induced a predominant IFN-γ response.

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Abbreviations: DTH, delayed-type hypersensitivity; LN, lymph node; mAb, monoclonal antibody; OA, ovalbumin; T-Ag, *Trypanosoma cruzi* trypomastigote antigen.

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MATERIALS AND METHODS

Animals

(BALB/c X A/J) F1 female mice (CAF) were bred in the inbred

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animal facilities of our department and immunized when 8–10 weeks old.

Antigens and reagents

Saponin, complete Freund's adjuvant (CFA), chicken egg albumin grade V (ovalbumin-OA), RPMI-1640, glutamine, 2-mercaptoethanol, penicillin-streptomycin, concanavalin A (Con A) and 2,2′-Azino-bis(3-Ethylbenzthiazoline-6-sulfonic acid) were bought from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum, virus and mycoplasma free, (FCS), was purchased from Guanandhy Ltd., Cuiabá, MT, Brazil. *T. cruzi* tissue-culture trypomastigote antigen (T-Ag) was prepared from Y strain parasites harvested from infected monolayers of LLC-MK2 cells (American Type Culture Collection (ATCC) CCL7·1) by six to eight cycles of freezing and thawing (FT), as previously described,[11] aliquoted and kept frozen until use. [^{3}H]thymidine ([^{3}H]TdR, specific activity 2 Ci/mM) and streptavidin-peroxidase were purchased from Amersham (Arlington Heights, IL).

Antibodies

The rat anti-mouse monoclonal antibodies (mAbs): anti-IL-10 (JES5-2A5), anti-CD4$^+$ (GK 1.5), anti-β galactosidase (GL-113-IgG1 isotype control) were purified by Sepharose–protein G chromatography from hybridoma cell culture supernatants. All antibodies for the cytokine assays were grown similarly, purified and biotinylated, as required, in our laboratory. Biotyn-labelled SXC-1 (anti-IL-10) as well as the hybridomas and recombinant standard cytokines were a kind gift from Dr Robert L. Coffman, DNAX Research Institute, Palo Alto, CA.

Immunization and cutaneous hypersensitivity testing

The antigens were diluted in 0·01 M sterile phosphate-buffered saline and emulsified vol/vol in CFA or mixed vol/vol with saponin solution (0·2% w/vol) just prior to injection. Fifty μl of the antigen–adjuvant mixture were injected into the subcutaneous space at each side of the tail base. Each animal received 100 μg ovalbumin (OA) or 5×10^7 FT parasites (T-Ag) administered alone or mixed with either adjuvant. Ten days after immunization, the mice were challenged by injecting 30 μl of aggregated OA solution (20 mg/ml)[12] or 20 μl of T-Ag with 2×10^7 parasites, intradermally, into one of the hind footpads. Footpad swelling was monitored for the next 48 hr with a metric gauge-caliper (precision 0·01 mm) and expressed as the increase in thickness relative to the uninjected contralateral paw. Results are presented as the arithmetic mean ± SEM (n = 5).

Lymph node cell cultures

Twelve days after immunization, cells from the draining lymph node (LN) (superficial inguinal and periaortic) were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, 0·05 M 2-mercaptoethanol, penicillin/streptomycin (100 μg of each) and 10% FCS in 96-well flat-bottomed plates at a density of 4×10^5 cells/200 μl/well for the proliferation assays or at 3×10^6 cells/ml in 24-well plates for the collection of 24 hr or 72 hr supernatants. The cultures were stimulated with T-Ag (2×10^6/ml), OA (100 μg/ml) or Con A 2·5 μg/ml, or just maintained without stimulus in tissue culture medium. The mAbs JES-2A5 or GL 113 were added at a concentration of 20 μg/ml at the beginning of the cultures; mAb GK 1.5 was added at 3·3 μg/ml. At these concentrations none of the mAbs exhibited toxicity to the cell cultures. [^{3}H]TdR (0·5 μCi/well) was added to the proliferation assays 18 hr before cell harvesting at 72 hr of culture. Radioactivity was measured by scintillation counting and the data presented as the arithmetic mean (c.p.m.) of triplicate cultures and the SD.

Cytokine assays

Cytokine levels in the culture supernatants were measured by two-site sandwich enzyme-linked immunosorbent assay (ELISA) using the following mAb pairs of which the second cited was biotinylated: IFN-γ, XMG 1.2 and AN18; IL-10, JES-2A5 and SXC-1; interleukin-4 (IL-4), 11 B 11 and BVD6 24G2. Standard curves were obtained with recombinant mouse cytokines. The reaction was developed with peroxidase-labelled streptavidin followed by the substrate mixture containing hydrogen peroxide and ABTS as chromogen. The sensitivity of the IL-4 assays was increased by using the ELAST ELISA amplification system purchased from DuPont NEN, Wilmington, DE. The supernatants were tested in serial two-fold dilutions and the results expressed as the arithmetic mean of duplicate determinations. The SD did not exceed 20% of the mean.

RESULTS

Immunization with saponin stimulates antigen-specific DTH and T-cell proliferation of draining LN cells

Mice immunized subcutaneously with *T. cruzi* antigen or with OA using saponin as adjuvant displayed strong DTH reactions when challenged in the footpad with the corresponding antigens 10 days after the primary immunization. The levels of the 24 hr footpad reactions to each antigen in saponin-immunized mice were comparable with those expressed after immunization with either antigen in CFA (Fig. 1, a versus c and b versus d). OA-elicited reactions were still high at 48 hr in mice immunized with this antigen plus saponin or CFA (Fig. 1b, 1d) when compared with control non-immunized mice; T-Ag elicited reactions in T-Ag plus adjuvant-immunized mice (Fig. 1a, 1c) had sharply declined by 48 hr. This result suggests that the persistence of the reaction until 48 hr depended on the nature of the antigen and not of the type of adjuvant used for immunization. Histopathology of the 24 hr reactions to OA or T-Ag of saponin-immunized mice showed a predominant mononuclear cell infiltrate with few polymorphonuclear cells (data not shown). Lymph node cell cultures from saponin plus antigen (OA or T-Ag)-immunized mice showed strong proliferative antigen-specific responses (Fig. 2(a), 2(b)). The magnitude of these responses was similar (OA) or higher (T-Ag) than those observed in LN cell cultures from CFA plus antigen-immunized mice. Antigen-specific proliferation by LN cells in either immunization condition (CFA or saponin plus OA or T-Ag) was mainly CD4 activation-dependent, as the addition of the mAb GK1.5 to the cultures reduced proliferation by 70–80% (data not shown). On the other hand, Con A-stimulated cultures showed no reduction of proliferation by treatment with mAb GK 1.5 indicating that this mAb was not toxic to cell cultures.

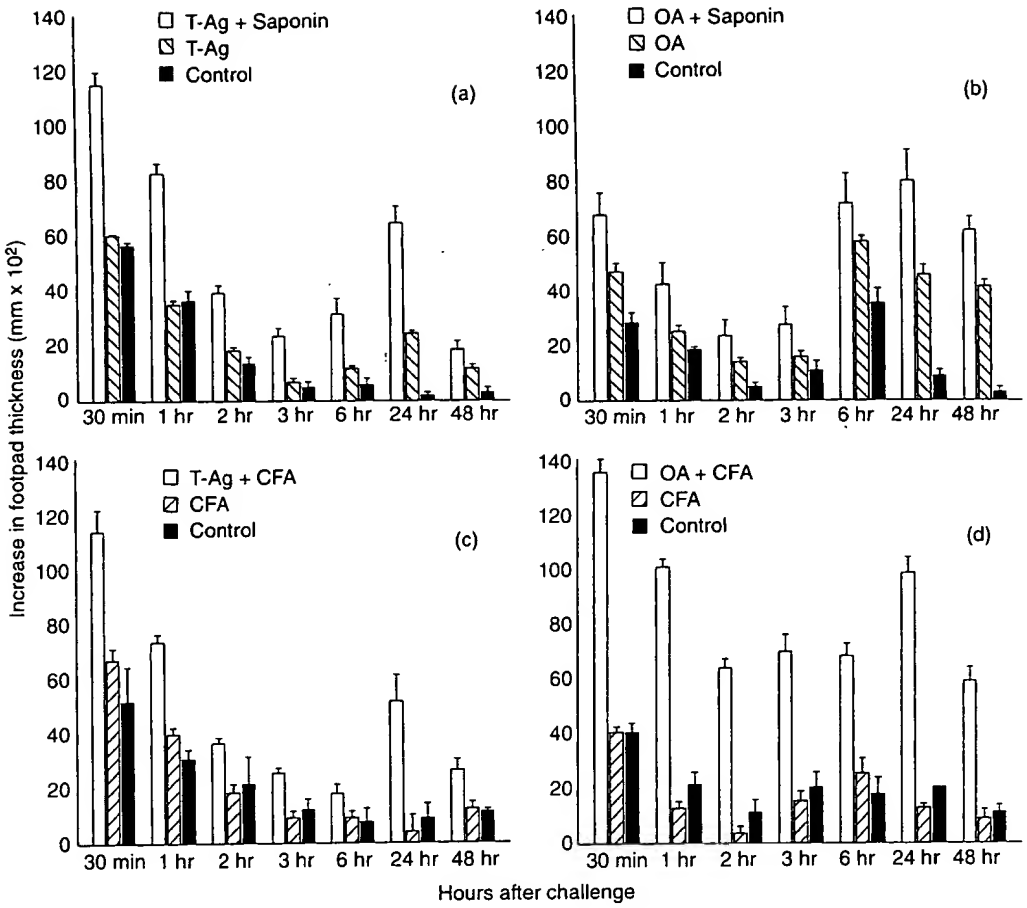

Figure 1. Cutaneous hypersensitivity responses in mice immunized with saponin plus T-Ag (a) or OA (b) or with CFA plus T-Ag (c) or OA (d). Ten days after immunization the corresponding challenging antigen was injected intradermally into one of the hind footpads. (□) immunization with adjuvant plus antigen; (▨) immunization with antigen minus adjuvant; (▨) injection of CFA minus antigen; (■) control normal mice injected in the footpad with the challenge antigen. Values represent the arithmetic mean ± SE of five mice. Two further experiments gave similar results.

The data so far were indicative that saponin was as efficient as CFA at stimulating antigen-specific cellular immune responses. We investigated next the cytokines produced *in vitro* by cells from mice immunized with either adjuvant.

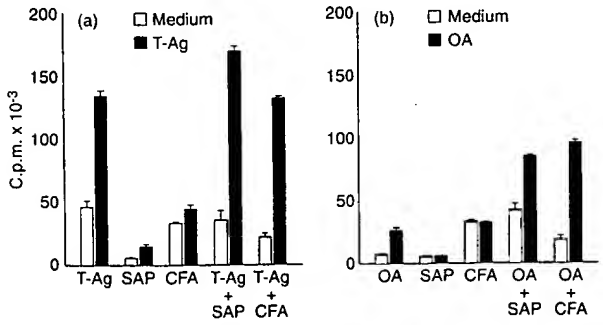

Figure 2. Proliferative responses of lymph node cells from mice immunized with saponin or CFA plus T-Ag (a) or OA (b). The responses from mice immunized with antigen only or with adjuvant only are also shown. The cultures were stimulated for 72 hr with antigen (■) or kept in culture medium without stimulation (□). Values are expressed as the arithmetic mean of c.p.m. from triplicate cultures ± SD. The results are representative of a set of three independently run experiments.

CFA-stimulated IFN-γ production and suppresses IL-10 production in mice immunized with OA or T-Ag

Cells from mice immunized with OA without adjuvants produced very low levels of IFN-γ in antigen or Con A-stimulated cultures and moderate amounts of IL-10. Cells from mice immunized with T-Ag without any adjuvant and stimulated *in vitro* with the same antigen produced higher amounts of IL-10 from OA-immunized mice and comparably low amounts of IFN-γ (Tables 1 and 2). Immunization with OA or T-Ag together with CFA as adjuvant primed the LN cells to enhanced production of IFN-γ upon *in vitro* stimulation with the homologous antigen: 12 and 20-fold higher levels of IFN-γ were obtained in cultures from mice immunized with T-Ag or OA plus CFA than without the adjuvant (Table 1). Production of IFN-γ in both antigen systems was CD4 activation-dependent, as treatment of the cultures with mAb GK 1.5 almost completely abolished antigen-specific IFN-γ production. The increased IFN-γ production seen in LN cell cultures from CFA plus antigen-immunized animals was accompanied by a concomitant marked decrease in the production of IL-10 as compared with cultures from mice immunized only with the antigen, T-Ag or OA (Table 2). IL-4 production in cultures from CFA plus antigen-immunized mice was low in antigen

Table 1. IFN-γ production by lymph node cells from mice immunized with CFA or saponin (SAP) and T-Ag or OA

Immunization	Stimuli in culture		
	T-Ag or OA*	T-Ag or OA + GK 1.5	Con A
T-Ag	2·8†	< 1·6	3·2
OA	< 1·6	< 1·6	2·6
SAP + T-Ag	4·0	< 1·6	6·1
SAP + OA	< 1·6	< 1·6	3·2
CFA + T-Ag	33·5	2·7	19·5
CFA + OA	44·7	< 1·6	16·6

* The cultures were stimulated *in vitro* with the homologous antigen used for immunization and the supernatants were harvested after 72 hr of culture. The anti-CD4 mAb GK 1.5 was added to antigen-stimulated cultures at the start of culture at a concentration of 3·3 μg/ml.

† IFN-γ concentrations are expressed in ng/ml; unstimulated cultures produced less than 1·6 ng/ml of IFN-γ.

Table 3. IL-4 production by lymph node cells from mice immunized with CFA or saponin (SAP) and T-Ag or OA

Immunization	Stimuli in culture		
	Medium	T-Ag or OA*	Con A
SAP + T-Ag	< 2·0†	6·0	78·0
SAP + OA	< 2·0	6·0	47·0
CFA + T-Ag	< 2·0	< 2·0	16·0
CFA + OA	< 2·0	3·0	17·0

* The cultures were stimulated *in vitro* with the homologous antigen used for immunization and the supernatants were harvested after 24 hr of culture.

† IL-4 concentrations are expressed in pg/ml.

and in Con A-stimulated cultures (Table 3). Production of IFN-γ and IL-10 was below detection levels in unstimulated LN cultures from mice injected only with CFA. Con A stimulation of these cultures yielded 5·6 U/ml IL-10 and 27·1 ng/ml IFN-γ, neither one suppressed by GK 1.5 treatment. Unstimulated LN cultures from normal mice did not yield measurable IFN-γ levels whereas upon Con A stimulation, IFN-γ production was 17·5 ng/ml and IL-10 was not detected.

Taken together, these results indicate that CFA strongly directs the priming of antigen-specific T cells towards a Th1 type of cytokine response.

Saponin stimulates IL-10 production in mice immunized with OA or with T-Ag

Immunization with T-Ag or OA and saponin as adjuvant resulted in a quite different pattern of cytokine production from the one obtained using CFA as adjuvant. Upon secondary antigen stimulation *in vitro*, LN cells from either T-Ag or OA plus saponin-immunized mice secreted high amounts of IL-10 in culture (Table 2). Saponin further increased IL-10 production by LN cells immunized with T-Ag, an antigen that already induced high IL-10 production when injected without adjuvant. However, saponin plus OA immunization markedly increased IL-10 production to OA, that by itself stimulates only moderate amounts of IL-10 (Table 2). The strong inhibition of antigen-specific IL-10 production by the mAb GK 1.5 indicated its predominant origin from $CD4^+$ activated cells (Table 2). The highest levels of antigen-specific IL-10 production were seen when saponin was used as an adjuvant to T-Ag; in this condition of immunization, elevated IL-10 was also seen in polyclonally stimulated cultures. Production of IFN-γ was low in antigen-specific or in Con A-stimulated cultures from OA or T-Ag plus saponin immunized mice (Table 1). Antigen-specific production of IL-4 in cultures from saponin plus antigen-immunized mice was low and did not differ from the levels obtained from antigen plus CFA-immunized mice (Table 3). However, higher levels of IL-4 in cultures from saponin versus CFA-immunized mice were obtained in supernatants from Con A-stimulated cultures (Table 3). Production of IFN-γ was below detection levels in unstimulated LN cultures from mice injected only with saponin; IL-10 production was low at 7·5 U/ml. However, upon Con A stimulation of these cultures IFN-γ levels reached 44·7 ng/ml and IL-10 was 5·3 U/ml. This suggests

Table 2. IL-10 production by lymph node cells from mice immunized with CFA or saponin (SAP) and T-Ag or OA

Immunization	Stimuli in culture			
	Medium	T-Ag or OA*	T-Ag or OA + GK 1.5	Con A
T-Ag	11·5†	50·2	7·9	13·6
OA	4·9	17·4	< 3·1	10·0
SAP + T-Ag	6·3	80·0	15·7	29·3
SAP + OA	7·3	59·8	6·6	10·5
CFA + T-Ag	< 3·1	15·4	< 3·1	5·2
CFA + OA	< 3·1	8·4	< 3·1	5·1

* The cultures were stimulated *in vitro* with the homologous antigen used for immunization and the supernatants were harvested after 72 hr of culture. The anti-CD4 mAb GK 1.5 was added to antigen-stimulated cultures at the start of culture at a concentration of 3·3 μg/ml.

† IL-10 concentrations are expressed in U/ml.

Table 4. Effect of IL-10 on Con A-induced lymph node cell proliferation

Immunization	Con A-induced proliferation (c.p.m. × 10^{-3}) Medium	Anti-IL-10 mAb*
T-Ag	42·35 ± 2·30†	113·63 ± 5·42
SAP‡	136·42 ± 7·80	133·24 ± 13·35
SAP + T-Ag	62·63 ± 5·18	115·13 ± 1·84
Normal	181·14 ± 2·86	166·95 ± 16·95
OA	121·16 ± 9·06	102·83 ± 10·25
SAP + OA	66·12 ± 3·45	61·13 ± 1·26

* The anti-IL-10 mAb JES-2A5 was added at 20 μg/ml at the start of culture.

† Mean of triplicates ± SD.

‡ SAP, saponin.

that saponin, by itself, stimulates moderate production of IL-10 but does not establish a dominant Th2-type millieu in the draining LN as indicated by the concomitant high IFN-γ production upon polyclonal stimulation.

Altogether, these results indicate that immunization with antigen using saponin as adjuvant stimulates the priming of antigen-specific T cells towards a pattern of high IL-10 and low IFN-γ production.

IL-10 produced by LN cells from saponin plus T-Ag immunized mice inhibits antigen-specific IFN-γ production and Con A-stimulated proliferation

The possibility that the high levels of IL-10 produced by LN cell cultures from T-Ag or T-Ag plus saponin-immunized mice would negatively control IFN-γ production in the same cultures was tested by treating the cultures with a neutralizing anti-IL-10 mAb and measuring IFN-γ production. Cultures treated with the 2A5 mAb produced 26·8 and 38·8 ng/ml IFN-γ in contrast with 2·8 and 4·0 ng/ml IFN-γ of untreated cultures.

Although immunization with saponin as adjuvant was very efficient at inducing T-Ag or OA specific proliferative responses by LN cells *in vitro* (Fig. 2), Con A stimulation of the same cultures or cultures from T-Ag immunized mice consistently induced less proliferation than in control cultures (Table 4). The possibility that IL-10 produced in these cultures was inhibitory to Con A proliferation was tested by treating the cultures with the neutralizing mAb 2A5. As shown in Table 4, treatment with the anti-IL-10 mAb restored Con A-induced proliferation to the levels of cultures from control mice inoculated only with saponin. However, no restoration of Con A proliferation was seen in cultures from OA plus saponin-immunized mice suggesting that, for this antigen, other mechanisms might be responsible for suppression of mitogen-induced cell proliferation by antigen-primed lymphocytes.

DISCUSSION

The present study shows that immunization with saponin as adjuvant stimulates the production of IL-10 by antigen-primed T lymphocytes. To our knowledge, this is the first time that the selective use of an adjuvant has been shown to stimulate IL-10 responses.

Although IL-10 can be produced by many cell types including macrophages, among lymphocytes only Th2 type cells and a subset of $CD4^+$ thymocytes produce this cytokine (reviewed in 13). Inhibition of IL-10 production by anti-CD4 mAb indicated that T cells were the major source of IL-10 in saponin plus T-Ag or OA-immunized mice. The antigen-stimulated expression of IL-10 in culture is considered to correlate well with the induction of Th2-type responses *in vivo*.[13] Although production of IL-4 to secondary antigen stimulation *in vitro* was not augmented in LN cell cultures from saponin-immunized mice when compared with LN cell cultures from CFA-immunized mice, the former produced more IL-4 upon Con A stimulation. On the other hand, Th2 $CD4^+$ cell lines producing high levels of both IL-10 and IL-4 were easily derived from these LNC cultures after just a single round of *in vitro* stimulation followed by expansion in EL4 conditioned medium.[14] Taken together, these results indicate that immunization with saponin favoured the *in vivo* priming towards a Th2-type response. Although IL-4 is the major drive towards Th2 differentiation *in vitro*, there is evidence that IL-10 decreases priming for IFN-γ production and thus favours the development of Th2-type secreting cells in the presence of moderate amounts of IL-4.[15,16] That IL-10 was indeed down-regulating IFN-γ production by LN cells from saponin-immunized mice was confirmed by the increased production of IFN-γ in cultures treated with a neutralizing anti-IL-10 antibody. This result is indicative of the presence of an antigen-specific IFN-γ-secreting population in the LN from saponin-immunized mice.

That immunization with saponin also primed Th1-type cells in addition to Th2 is further supported by the expression of DTH by saponin-immunized mice. Actually, saponin was comparable to CFA at inducing cutaneous DTH to both *T. cruzi* antigen and to OA. Inasmuch as DTH expression was only partially inhibited in mice treated systematically with recombinant (r)IL-4 and rIL10[17] or with rIL-10,[18] and as only a few sensitized $CD4^+$ cells are required to trigger DTH at a skin site challenged with specific antigen,[19] expression of DTH might persist in a situation of activation of both Th1 and Th2-type responses.

Originally, we became interested in saponin because immunization with a purified parasite glycoprotein associated with this adjuvant was reported to induce strong cellular immunity and protective immunity to *T. cruzi*.[7] However, we were unable to obtain any protective immunity to a live parasite challenge in mice immunized with saponin and the *T. cruzi* antigen preparation that we used (data not shown). As our protocol of immunization stimulated a dominant Th2 pattern of cytokine response, the lack of protective immunity was not surprising. T-cell lines that produce high levels of IL-10 and IL-4 have been shown to aggravate the course of infection[14] and at the effector cell level, recombinant IL-10 inhibits IFN-γ-mediated macrophage activation for intracellular killing of *T. cruzi in vitro*.[20,21] On the other hand, bacillus Calmette-Guérin (BCG) immunization partially protects mice from *T. cruzi* infection[22] and thus interpretation of partial protection data obtained with CFA plus T-Ag immunization is rendered difficult.

The mechanisms by which saponin exerts its adjuvant effects are largely unknown. Attachment to the antigen is not essential for the adjuvant effect[23] that is probably mediated through complexing and cholesterol present in the membrane cells of the host immune system.[24] Whether saponin (like lipopolysaccharide) would activate macrophages to IL-10 synthesis and thus favour the initial development of Th2 cells is a matter of speculation. Cholera toxin (CT) is considered to be a strong stimulator of Th2-type cytokine responses.[25] However, the reported increase in IL-10 production after CT immunization accompanied the enhanced synthesis of both Th1 and Th2-type cytokines.[26]

The pattern of antibody isotypes synthesized in response to immunization with adjuvants might provide indirect information on their ability to stimulate preferentially a Th1 or Th2-type response. IgG1 and IgE antibody responses are associated with Th2 stimulation whereas IgG2a is associated with Th1-type responses. Among a panel of different adjuvants tested, the saponin adjuvant Quil A, originally purified from Quillaia bark saponins[1] and alum hydroxide resulted in the predominant production of IgG1 antibodies whereas CFA stimulated both IgG1 and IgG2.[8] However, saponins also induced significant levels of IgG2a and IgG2b as well as IgG1 antibodies in other immunization protocols.[4–7] Saponin adjuvants are also potent inducers of anti-OA $CD8^+$ cytotoxic T-lymphocyte responses and in association with alum potentiated the proliferative and antibody responses to human immunodeficiency virus (HIV) recombinant proteins.[27,28] No obvious correlation was found between the induced isotypes (IgG1, predominant, and IgG2) and the serum cytokine profile in mice immunized with an influenza vaccine associated with Quil A saponin fraction LTC[6] (Isotec Ab, Luleå, Sweden) or in sheep immunized with Quil A saponin and OA.[29] Altogether, these results suggest that saponins can stimulate both IgG1 and IgG2 antibody responses, which correlates with a stimulation of both Th2 and Th1-type responses. However, by comparison with muramyl dipeptide and $Al(OH)_3$ only Quil A was effective at stimulating high IFN-γ production to the HIV glycoprotein 120 (gp 120).[5] In addition, complexes comprising Quil A, lipids and influenza virus antigen were efficient inducers of Th1-type cytokine responses.[30] Quil A saponin also augmented antibody responses to T-independent antigens.[31] The use of selected saponin fractions versus non-purified Quil A, the nature of the antigen and the chosen immunization schedule might alter the balance towards either type of response.

Our results were obtained using unfractionated saponins extracted from *Quillaia* as adjuvant and also show stimulation of Th1 and Th2-type responses to both OA and *T. cruzi* antigen. The cytokine pattern was marked by a dominant IL-10 response. IL-10-mediated suppression of Con A proliferation, originally described by Shevach *et al.*,[32] was observed in cultures from saponin plus T-Ag immunized mice; *in vitro* treatment with anti-IL-10 mAb restored mitogen-induced proliferation and increased IFN-γ production. CFA, in accordance with previous published studies,[10,29] was a strong stimulus to IFN-γ production in both antigen systems and drove the immune response to *T. cruzi* antigen towards a high IFN-γ and diminished IL-10 production.

We found immunization with saponin to be a useful protocol to prime the immune system towards a Th2 response. Given the complex regulatory roles exerted by IL-10 in the immune response, our results stress the importance of evaluating its production when assessing the effects of adjuvants in immunization.

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EXR NOTES
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Title: SAPONIN ADJUVANT PRIMES FOR A DOMINANT INTERLEUKIN-10 PRODUCTION TO OVALBUMIN AND TO TRYPANOSOMA-CRUZI ANTIGEN
Author(s): TADOKORO CE; MACEDO MS; ABRAHAMSOHN IA
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Abstract: The adjuvant activity of saponin for T-cell responses was evaluated and compared with that of complete Freund's adjuvant (CFA) in two antigen systems: a %%%lysate%%% of the protozoa Trypanosoma cruzi and ovalbumin (OA). Strong delayed-type hypersensitivity and T-cell proliferate responses, comparable with those stimulated by CFA, were observed for both antigens following immunization with saponin as

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Detection of antibodies to *Leishmania donovani* in animals in a kala-azar endemic region in eastern Sudan: a preliminary report

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Abstract

The prevalence of antibodies against *Leishmania donovani* in selected domestic and wild animal species in 2 villages in Sudan with active *L. donovani* transmission in humans was investigated. Screening of domestic animals (donkeys, cows, sheep, goats, camels and dogs) with the direct agglutination test (DAT) detected reaction rates above the cut-off titres in donkeys (68·7%), cows (21·4%) and goats (8·5%), and which were also found in wild rats (5·5%). Sera of sheep, camels and dogs had a weak agglutination reaction below the cut-off titre. Testing of the same sera by enzyme-linked immunosorbent assay (ELISA), against a lysate of *L. donovani* promastigotes, showed reaction rates above the cut-off optical density in cows (47·6%), goats (13·6%), and in rats (4·1%). No *Leishmania* parasite was isolated from spleen, liver, bone-marrow or spleen of Nile rats.

Keywords: *Leishmania donovani*, animal reservoirs, serology, antibodies, Sudan

Introduction

Visceral leishmaniasis (kala-azar) is a serious protozoan disease with an annual incidence rate of 1·5 to 2 million cases worldwide (DESJEUX, 1996). The disease is caused by *Leishmania* parasites and transmitted by sandflies. The infection can be anthroponotic, or zoonotic with animals serving as a reservoir. Epidemiological studies of visceral leishmaniasis worldwide had incriminated several animal species as reservoirs for *Leishmania* parasites. Dogs, jackals, rodents, and foxes have been identified as reservoirs for both *L. infantum* and *L. donovani* (AZAB *et al.*, 1984; IBRAHIM *et al.*, 1994). Although feeding of *Leishmania* vectors on other animal species, including cattle and equines, has been proven, the role of these animals in maintaining *Leishmania* parasites needs to be investigated.

Both the direct agglutination test (DAT; HARITH *et al.*, 1988, 1989; CHOUDHURY *et al.*, 1990, 1993) and enzyme-linked immunosorbent assay (ELISA; EL AMIN *et al.*, 1985) techniques are used for detection of *Leishmania* infection for diagnosis and epidemiological studies. Both techniques have high sensitivity and are specific in animals and humans (HARITH *et al.*, 1989; EL AMIN *et al.*, 1985).

Visceral leishmaniasis is endemic in Sudan with sporadic epidemics (SATTI, 1958; DE BEER *et al.*, 1990). Three endemic regions are identified in Sudan: Gadaref region in the east, Bantu-Malakal region in the south, and the El Fashir-Babanusa region in the west (SATTI, 1958; HOOGSTRAAL & HEYNEMAN, 1969). The epidemiology of visceral leishmaniasis in Sudan is not known except for the previous work of HOOGSTRAAL & HEYNEMAN (1969). The role of domestic and wild animals in transmission and maintenance of *L. donovani* needs to be investigated to allow effective control measures.

In this study we attempted to monitor the exposure of selected domestic and wild animal species to *L. donovani* infection, to determine their role in the epidemiology of visceral leishmaniasis in this region. Animals were screened for the presence of specific *L. donovani* antibodies as an indicator for exposure to infection. Attempts were also made to isolate the parasite from rat bone-marrow, liver, spleen and skin.

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Materials and Methods

Study site

Two villages (Bandigueo and Um Salala) within the visceral leishmaniasis endemic region in eastern Sudan were selected for this study. Epidemiological studies on humans have proven the active transmission of *L. donovani* in both villages, with an annual incidence rate of 5/1000 (ZIJLSTRA *et al.*, 1994). The 2 villages are located on the eastern bank of the seasonal Rahad River, which separates them from the Dinder National Park (shelter for wildlife), and they are about 5 km apart.

Serum samples

Blood was collected from the jugular veins of donkeys, cows, sheep, camels and goats and allowed to clot at room temperature (35°C). The serum was separated by centrifugation and then stored at −20°C. Blood spots were collected on Whatman No. 3 filter paper from dogs (ear veins) and from the heart of dissected wild rats.

Elution of sera from filter papers

Sera were eluted from filter paper as described by HARITH *et al.* (1989). Circles (0·5-cm diameter) of blood-spotted paper were cut and immersed in 1 mL elution buffer (0·9% NaCl, pH 7·4). The eluted sera were equivalent to a 1:20 dilution.

Screening of donkeys for Histoplasma capsulatum *infection*

The study villages are known to be endemic for equine ulcerative lymphangitis (*Histoplasma capsulatum* infection). To determine possible serological cross-reaction with *L. donovani*, all sampled donkeys were examined clinically for lymphatic ulceration and smears were made from suspected lesions for detection of the fungus.

Screening of camels for Trypanosoma evansi *infection*

Since the selected villages are known to be endemic for camel trypanosomiasis, all selected camels were screened for *T. evansi* infection. Wet blood smears were freshly made from each animal and examined directly under a light microscope for the presence of the parasite.

DAT antigen

Antigen was prepared from *L. donovani* (isolated from a Sudanese visceral leishmaniasis patient) as described by HARITH *et al.* (1988). Following trypsin treatment of the harvested promastigotes, the parasites were washed 5 times in Locke's solution (154-mM NaCl, 2-mM $CaCl_2$, 2-mM $NaHCO_3$). Promastigotes were fixed in 2% (w/v)

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formaldehyde in Locke's solution for 18 h. The fixed parasites were then washed with citrate–saline (0·15-M NaCl, 0·05-M sodium citrate, pH 7·4). The parasites were stained with Coomassie brilliant blue, washed twice in sodium citrate and then suspended at a concentration of $7·5 \times 10^7$/mL in 0·43% (w/v) formaldehyde in citrate–saline solution. The antigen was stored at 4°C until use.

Direct agglutination test (DAT)

The test was performed as described for detection of human visceral leishmaniasis (HARITH *et al.*, 1988). In brief, the agglutination activity was tested using V-shaped microtitre plates. Sera were tested in 2-fold serial dilutions (50 µL/well), starting at 1:50 dilution in gelatin diluent (0·15-M NaCl, and 2% (w/v) gelatin in distilled water supplemented with 0·1-M 2-mercaptoethanol). Antigen was added to the sera (50 µL/well) and the plates were gently rotated and then incubated at 26°C for 18 h.

The agglutination activity was detected visually and the titre was determined by identifying the serum dilution preceding the first sharp button identical to the positive control (human visceral leishmaniasis serum). The test was done in duplicate wells for each serum dilution. Both positive (from a confirmed visceral leishmaniasis patient) and negative human sera were used in each tested plate. Screening of agglutination reaction was done at a serum dilution of 1:200.

Determination of the agglutination and ELISA cut-off titres

Twenty animals of each tested species were selected as negative controls. All selected controls were raised in Khartoum city which is known to be free of visceral leishmaniasis. Sera were tested in 2-fold titration to determine the cut-off agglutination titre. The cut-off titre was calculated as the mean of the negative control animals plus 2 SD.

ELISA antigen

The antigen was prepared from promastigote cultures of *L. donovani* (Sudanese isolate) cultured in RPMI 1640 medium supplemented with 15% heat inactivated fetal bovine serum. Cultures were incubated at 26°C and the parasites were harvested at log phase by centrifugation at 400 *g* for 10 min at 4°C. The pellet was washed twice in ice-cold phosphate-buffered saline (PBS) and suspended in sterile PBS. The cell walls were lysed by repeated freezing and thawing in liquid nitrogen and a water-bath at 37°C. The cells were then subjected to ultrasonication for 5 min at 30-s bursts. The protein content of the lysate was determined by the BRADFORD method (1976).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was done as described by EL AMIN *et al.* (1985). In brief, each well of flat-bottomed Probind microtitre plates was coated with 0·5 µg parasite lysate in 100 µL carbonate–bicarbonate buffer (pH 9·6), and left at 4°C overnight. Excess antigen was decanted and the wells were blocked by 100 µL of 1% (w/v) bovine serum albumin (BSA) in PBS (pH 7·2) containing 0·05% Tween 20, for 1 h at room temperature. The test and control sera were then added in serial 2-fold dilution in diluent solution (1% BSA in PBS + 0·05% Tween 20) starting at 1:200, in duplicate wells. The reaction was allowed to proceed at room temperature for 1 h. The plates were then washed 3 times with washing buffer (PBS + 0·05% Tween 20). Secondary antibodies (100 µL of horseradish peroxidase-conjugated anti-species immunoglobulin antibodies, Sigma Chemicals) were added at 1:1000 in dilution buffer (100 µL/well) and incubated at room temperature for 1 h. The wells were then washed 3 times with the washing buffer and the reaction was visualized by addition of 100 µL/well of freshly prepared substrate, i.e., 0·4% (w/v) *o*-phenylenediamine (Sigma) in citrate buffer pH 5·0 plus 0·3% hydrogen peroxide. The colour was allowed to develop for 30 min and the reaction was stopped by addition of 50 µL of 20% H_2SO_4. The optical density was measured using an ELISA reader and recorded as the mean of the 2 duplicate wells for each dilution.

Parasite culture

Skin (ears and muzzles) of 113 captured wild *Arvicanthus niloticus* rats were carefully cleaned with methanol and autopsied for parasite culture. Spleen, liver and bone-marrow (from the femur) were aseptically cultured in NNN medium supplemented with 30% defibrinated rabbit blood. The agar was overlaid with RPMI 1640 supplemented with antibiotics (streptomycin and penicillin). The cultures were incubated at room temperature (26°C) and parasite growth was observed weekly. Subcultures were made in NNN, incubated at 26°C and observed for 6 weeks before being considered negative.

Results

An agglutination titre of 1:3200 was considered positive in this study, based on the reactivity of negative controls from non-endemic areas.

Sera collected from donkeys showed a high rate of agglutination activity against *L. donovani* Coomassie blue-stained antigens. Of the 96 tested animals 68·7% had a titre ≥1:3200, 40·6% ≥1:12 800, 13·5% ≥1:25 600, 10·4% ≥1:51 200, 6·2% ≥1:102 400, and 4·2% ≥1:204 800 (Table). None of the 5 donkeys diag-

Table. Agglutination of *Leishmania donovani* antigen by sera of different animal species in eastern Sudan in a direct agglutination test

Titre	Donkeys ($n = 96$)	Cows ($n = 42$)	Goats ($n = 59$)	Sheep ($n = 25$)	Camels ($n = 20$)	Rats ($n = 73$)
1:200	11	14	26	23	6	35
1:400	3	10	4	5	5	13
1:800	9	9	13	5	4	11
1:1600	7	0	11	2	5	10
1:3200*	19	2	5	0	0	3
1:6400	8	1	0	0	0	1
1:12 800	26	1	0	0	0	0
1:25 600	3	1	0	0	0	0
1:51 200	4	3	0	0	0	0
1:102 400	2	0	0	0	0	0
1:204 800	4	1	0	0	0	0

Values indicate the number of animals with agglutinating sera at the titre shown. See the text for methodological details.
*Cut off reaction titre.

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nosed with *H. capsulatum* infection had a significant *Leishmania* agglutinating activity.

Sera collected from cows had a high rate of agglutination activity when tested by DAT. Of the 42 tested sera 21·4% had an agglutination titre ≥1:3200, 14·3% ≥1:12 800, 11·9% ≥1:25 600, 9·5% ≥1:51 200 and 2·4% ≥1:204 800 (Table). Sera collected from goats showed a low agglutination rate; only 5 (8·5%) of the 59 tested sera had an agglutination titre ≥1:3200 (Table). Sera collected from sheep, camels and dogs had no agglutination activity (Table), and only 4 (5·5%) of the 73 sera collected from Nile rats agglutinated *L. donovani* antigen at dilutions above the cut-off level (Table).

Only 3 (4·1%) rats were reactive in ELISA (OD > 0·60), and none of the agglutinating sera reacted with *L. donovani* lysate in ELISA. ELISA testing of sera collected from cows showed positive reactions (OD ≥ 0·8) in 20 samples. Eight of these samples had agglutination activity, while 12 did not agglutinate *L. donovani* antigen (Figure). ELISA testing of the goat sera identified 8 reactive sera (OD ≥ 0·8), whereas only 3 of the rat sera were reactive (OD ≥ 0·6) (Figure).

No parasite growth was observed from any cultured rat samples ($n = 113$).

Five donkeys had clinical and microscopy evidence of *H. capsulatum* infection. None of these 5 donkeys had serum that agglutinated *L. donovani* antigen. Three camels were confirmed to have camel trypanosomiasis.

Discussion

Although there is active transmission of visceral leishmaniasis in the studied area of eastern Sudan, the epidemiology of infection there is not yet known. We used both the DAT (HARITH *et al.*, 1988), and ELISA (EL AMIN *et al.*, 1985) to screen several selected domestic and wild animal species for the presence of anti-*Leishmania* antibodies as an indicator of exposure to infection by the parasite. The DAT cut-off titre was similar in all screened animal species (1:3200), in contrast to previous reports that determined a cut-off titre of 1:1600 in dogs infected with *L. infantum* (HARITH *et al.*, 1989). The screened domestic animal species (donkeys, cows, goats, sheep, camels and dogs) are commonly raised by villagers and are usually kept close to villagers' huts.

Screened donkeys had a high agglutination activity (68·7%), suggesting their exposure to the parasite, and many of them had a strong agglutination reaction, indicating the possibility of repeated exposure to *Leishmania* parasites. The fact that none of the sera from the 5 donkeys with *H. capsulatum* infection had agglutination activity against *L. donovani* antigen indicates the lack of cross-reaction with *Leishmania* parasites. To our knowledge, there is no report of infection of donkeys by other *Leishmania* parasites; however, feeding of *Leishmania* vectors on equines has been previously reported (WHO, 1991). Owing to the lack of anti-donkey immunoglobulin conjugates, the samples were not tested in the ELISA.

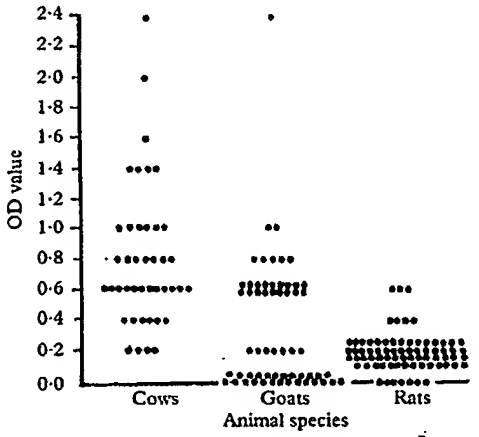

Figure. Reactivity of cow, goat and rat sera, from eastern Sudan, with *Leishmania donovani* extracts in an ELISA. Cuff-off titres: cows, optical density (OD) ≥ 0·8; goats, OD ≥ 0·8; rats, OD ≥ 0·6. See the text for methodological details.

Cows were the second most commonly reactive animal species. The high *Leishmania* agglutination reaction rate among cows (21·4%) suggests the exposure of these animals to *Leishmania* infection. A higher number of reactors was detected using the ELISA ($n = 20$), indicating a better detection ability of this technique. Eight of the tested animals were reactive in both DAT and ELISA.

A relatively low number of goats had positive agglutination reactions, only 5 of the 59 sampled. The same animals were reactive in ELISA indicating the specificity of the reaction. Although few reports have suggested the susceptibility of goats to *Leishmania* infection (WILLIAMS *et al.*, 1991), our results strengthen a possible role of these animals in the epidemiology of leishmaniasis in this region.

In this study area, few dogs were raised, and none of the 6 tested dogs had significant agglutination titres. Dogs were incriminated as reservoirs in southern Sudan as well as in the Mediterranean region for *L. donovani* and *L. infantum*, respectively (HOOGSTRAAL & HEYNEMAN, 1969; HARITH *et al.*, 1989). With the expectation of an infection rate of 5% among dogs, more dogs need to be tested to investigate their role in the transmission of leishmaniasis.

Interestingly, none of the 20 tested camel sera agglutinated *Leishmania* antigen. Three of these animals were confirmed cases of camel trypanosomiasis, indicating the lack of cross-reaction between *T. evansi* and *L. donovani*; however, more animals need to be tested. Similarly, none of the sheep sera ($n = 25$) agglutinated *L. donovani* antigen.

A. niloticus was the only captured rodent around the 2 villages. These rodents live mainly on the banks of the river and few live on trees around the huts. Only 4 rats (5·4%) out of the 73 captured had agglutination titre ≥1:3200, while the ELISA test identified 3 reactors. None of the agglutinating sera had reactivity in ELISA.

Parasite cultures from skin, spleen, and bone-marrow samples collected from captured rats were unsuccessful. The failure to grow the parasite in this study is not due to deficiency in our culture technique since samples collected from human visceral leishmaniasis cases are routinely grown in our laboratory using the same technique and culture media. More rats need to be examined to confirm our results since the infection rate in rats was reported to reach 1% in previous studies (HOOGSTRAAL & HEYNEMAN, 1969).

The high proportion of animals with positive agglutination reactions among donkeys, cows and goats suggests the exposure of these animals to *L. donovani* infection, and needs further investigation. The parasite needs to be isolated and typed in order to confirm the role of these animals in maintenance and transmission of visceral leishmaniasis. The variability of the sensitivity of DAT in different animal species has to be considered in future testing.

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Book Review

Haematology: Biomedical Sciences Explained Series. C. J. Pallister. Oxford: Butterworth Heinemann, 1999. x+262pp. Price £19.99. ISBN 0-7506-2457-4

This volume is aimed at undergraduates studying for a BSc in Biomedical Sciences. It reviews clinical and laboratory haematology, with emphases on the physiology and pathophysiology of the blood. It covers normal haemopoiesis, anaemias, malignant disorder of the blood, normal haemostasis, haemorrhagic disorders and thrombotic disorders. (Blood transfusion is the subject of another volume in the series.) The text is clear, generally accurate, inclusive and up to date. It is somewhat marred by misprints, for example, the tables of rhesus genotypes and of the FAB classification of myelodysplastic syndromes are confused. This book will be valuable in the preparation of medical laboratory scientists and it should enjoy popularity amongst its target audience in the British Isles. In many developing countries, medical laboratory workers have not been given the esteem commensurate with their responsibilities, but some universities are introducing belatedly BSc courses in Biomedical Sciences. This volume could be adopted usefully as a course-book in developing countries, but some caution is needed.

When the most important causes of anaemia in the tropics are considered, malarial anaemia is given only one-and-a-half lines of text. Deficiencies of iron and folate are covered adequately, although the nutritional impact of prolonged lactation is not mentioned. The thalassaemias receive detailed descriptions, except for an inaccurate map of their distribution: it is not true that α^+ thalassaemia is most common in American Blacks, as this forgets Africa itself and some Asian and Oceanic populations. The account of haemoglobin S is cursory, whereas haemoglobins showing instability or altered oxygen affinity are fully described despite their rarity. The statement that sickle-cell anaemia may be co-inherited with hereditary persistance of fetal haemoglobin is one rare error. HIV and tuberculosis as important causes of anaemia of chronic disorders are not mentioned. South-East Asian ovalocytosis, HTLV-I in Africa, and the unusual epidemiology of chronic lymphocytic leukaemia in tropical Africa are left out. More positively, the author notes correctly that snake bite is the commonest cause of disseminated intravascular coagulation (DIC) worldwide, that Africans have a high incidence of myeloma, that glucose 6-phosphate dehydrogenase deficiency is a common cause of neonatal jaundice and that Hodgkin's disease has a peak incidence in childhood in developing countries.

Colloquialisms relieve tension in readers of closely argued texts, but if English is not their first language they may have difficulty in understanding DIC, where activation of complement is said 'to cap it all', and 'once triggered, ... can rapidly spiral out of control'.

Despite these criticisms, this volume can be recommended to organizers and students of BSc (Biomedical Sciences) courses in the developing countries. It may be hoped that a second edition will have a more global approach which would enhance its usefulness in both developing and industrialized countries.

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The book can be ordered from: Heinemann Publishers Oxford, P.O. Box 382, Halley Court, Jordan Hill, Oxford OX2 8RU, UK; fax +44 (0) 1865 314091.

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AUTHOR: REED S G (Reprint); CARVALHO E M; SHERBERT C H; SAMPAIO D P; RUSSO D M; BACELAR O; PIHL D L; SCOTT J M; BARRAL A; ET AL
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RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: T cell responses are correlated with recovery from and resistance to %%%leishmaniasis%%%. Antigens of %%%Leishmania%%% chagasi were evaluated by determining their ability to elicit in vitro proliferation and cytokine production in peripheral blood lymphocytes and in T cell lines and clones from patients with histories of %%%leishmaniasis%%% or Chagas' disease. Antigens tested were selected by their reactivity with patients antibodies. Several of the antigens induced proliferative responses in peripheral blood lymphocytes from patients recovered from visceral or cutaneous %%%leishmaniasis%%% or with chronic Chagas' disease. Two purified glycoproteins, 30 and 42 kD, were consistently among the most effective in eliciting high proliferative responses and Il-2 production. Lymphocytes from a recovered visceral %%%leishmaniasis%%% patient were used to produce T cell lines against either the 30- or 42-kD antigen. Each of the lines responded to both of these antigens as well as to crude %%%leishmania%%% %%%lysate%%%. CD4+ T

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CHARACTERIZATION OF A PROTEIN FRACTION CONTAINING CYTOCHROMES B AND C-1 FROM MITOCHONDRIA OF %%%LEISHMANIA%%%-TARENTOLAE
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ABSTRACT: A soluble red band fraction was obtained from %%%Leishmania%%% tarentolae cells by sucrose gradient sedimentation of a Triton X-100 %%%lysate%%%. Spectral

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Vaccination of Balb/c mice against experimental visceral %%%leishmaniasis%%% with the GP36 glycoprotein antigen of %%%Leishmania%%% donovani

De Souza, EP; Bernardo, RR; Palatnik, M; De Sousa, CBP
Instituto de Microbiologia, 'Prof. Paulo de Goes', Universidade Federal do

EXPERIMENTAL PARASITOLOGY 68, 443–449 (1989)

Characterization of a Protein Fraction Containing Cytochromes *b* and c_1 from Mitochondria of *Leishmania tarentolae*

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SHAW, J. M., AND SIMPSON, L. 1989. Characterization of a protein fraction containing cytochromes *b* and c_1 from mitochondria of *Leishmania tarentolae*. *Experimental Parasitology* 68, 443–449. A soluble red band fraction was obtained from *Leishmania tarentolae* cells by sucrose gradient sedimentation of a Triton X-100 lysate. Spectral analysis indicated that cytochrome *b* was present in the red band: the reduced minus oxidized difference spectra revealed absorption maxima at 562, 527, and 431 nm at room temperature and 562, 530, and 422 nm at 77K. In addition, a 28-kDa protein was identified in this fraction which retained heme-associated peroxidase activity even after denaturation on SDS–polyacrylamide gels. The amino acid composition of this protein showed a strong similarity to cytochrome c_1 of both bovine and yeast. © 1989 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania tarentolae;* Kinetoplastid; Mitochondria; Cytochrome *b;* Cytochrome c_1; Heme protein; Sodium dodecyl sulfate (SDS); Phenylmethylsulfonyl fluoride (PMSF); 3,3′,5,5′-Tetramethylbenzidine (TMBZ); Hydrogen peroxide (H_2O_2); Phenylisothiocyanate (PITC); Nanometers (nm); Deoxyribonucleic acid (DNA); Ribonucleic acid (RNA).

INTRODUCTION

The kinetoplastid protozoa are a group of lower eukaryotes that contain a single, large mitochondrion in each cell. In all of the kinetoplastids, except for the blood stream African trypanosomes, the mitochondrion appears to have a fully functional respiratory system (Hill 1976; Martin and Mukkada 1979; Hart *et al.* 1981). Evidence for the presence of cytochrome *b*, cytochrome aa_3, and possibly cytochrome *o* has been obtained by spectral analysis (Hill and Cross 1973; Hill 1976; Martin and Mukkada 1979). The complete (or partial) amino acid sequence of cytochrome c_{555} has been determined for six kinetoplastids representing four different genera (Hill *et al.* 1971a,b; Pettigrew 1972; Rassam and Dawood 1986), and a cDNA encoding the apoprotein was recently cloned from *Trypanasoma brucei* and was partially sequenced (Torri and Hajduk 1988). To date, however, cytochrome *c* is the only respiratory protein that has been purified and biochemically characterized from these organisms.

This paper describes the properties of cytochromes *b* and c_1 found in a mitochondrial protein fraction of the kinetoplastid *Leishmania tarentolae*. In addition, a 28-kDa heme-binding protein has been isolated from this fraction and identified by amino acid composition analysis as the nuclear-encoded cytochrome c_1.

MATERIALS AND METHODS

Cell culture. L. tarentolae (UC strain) was grown in Difco brain–heart infusion medium supplemented with 10–20 μg/ml hemin at 27°C as described previously (Simpson and Braly 1970). Cells were grown to midlog phase and were used immediately for the isolation of mitochondria.

Mitochondrial isolation. Mitochondria were isolated from midlog phase cells by the Renografin flotation method (Simpson and Braly 1970; Simpson and Simpson 1978). Mitochondria were either used immediately or stored as a pellet at −70°C prior to solubilization.

Preparation of mitochondrial lysate. Mitochondria were solubilized by homogenization at 0°C in 0.2 *M* Tris–Cl, pH 8.0, 0.4 *M* KCl, 0.2 *M* sucrose, 35 m*M*

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$MgCl_2$, 25 m*M* EGTA, 2% Triton X-100, 1 m*M* PMSF, and 1.5 m*M* 2-mercaptoethanol. Subsequent fractionation over a 35–45% sucrose gradient for 24 hr at 60,000 rpm yielded two protein fractions which were visualized as a lower red band and an upper green band. These bands were recovered by dripping.

Gel electrophoresis. Protein samples were electrophoresed on 10–20% acrylamide gradient gels using a buffer system described by Laemmli (1970) or on 15% acrylamide/8 *M* urea/SDS gels as described by Ching and Attardi (1982).

Spectral analysis. The dithionite-reduced minus ferricyanide-oxidized difference spectra of sonicated, whole mitochondria and the red band fraction were measured with a Cary Model 219 double beam spectrophotometer at room temperature. The red band fraction was air-oxidized and ferricyanide was omitted from this sample. Whole mitochondria were sonicated in a buffer containing 0.25 *M* sucrose, 20 m*M* Tris-Cl, pH 7.9, and 2.0 m*M* EDTA. The scan rate was 1 nm/sec between 400 and 700 nm and the scale sensitivity was adjusted according to the absorbance.

TMBZ staining of polyacrylamide gels. The red band protein fraction was separated on 10–20% polyacrylamide gels prepared as described by Laemmli (1970). After electrophoresis, the gel was briefly rinsed in distilled water and stained with TMBZ for peroxidase activity using a procedure described by Thomas *et al.* (1976). A 6.3 m*M* TMBZ (Aldrich Chemical Co.) solution was freshly prepared in methanol. Immediately before use, three parts of the TMBZ solution were mixed with seven parts of 0.25 m*M* sodium acetate, pH 5.0. The gel was immersed in this solution for 1–2 hr in the dark with constant agitation. H_2O_2 was added to a final concentration of 30 m*M*. After the stain had developed (30 min to 1 hr), the gel was immersed in isopropanol:0.25 *M* sodium acetate, pH 5.0, at a ratio of 3:7 to clear the gel background and enhance the staining intensity. The gel was then photographed and stained for protein with Coomasie brilliant blue to facilitate gel isolation of the heme-binding protein.

Amino acid analysis of the 28-kDa heme-binding protein. After staining with Coomassie blue, the 28-kDa heme-containing protein band was excised from the gel, was soaked in several changes of distilled water, and the protein was eluted by electrophoresis into dialysis tubing using a buffer system described by Hunkapiller *et al.* (1983). After dialysis in 0.02% sodium dodecyl sulfate, 0.01 *M* NH_4HCO_3, the sample was dried and used for amino acid analysis. The use of a glycine buffer in this gel system results in slightly elevated values for glycine in the subsequent amino acid analysis.

The amino acid composition of the eluted protein was determined after 18, 24, and 65 hr of hydrolysis in 6 *N* HCl at 110°C using the Waters Picotag system and precolumn derivatization with PITC. Duplicate analyses were performed on each hydrolysate.

Results

Spectral analysis of whole mitochondrial particles. The dithionite-reduced minus ferricyanide-oxidized difference spectra of whole, sonicated mitochondria from *L. tarentolae* are shown in Fig. 1. The following components of the mitochondrial respiratory chain are represented in the spectrum: cytochrome aa_3 with α absorption maximum at 606 nm, a large amount of cytochrome *b* with α band at 560 nm, the β band at 530 nm, and the Soret γ band at 430 nm, and the flavins which appear as a trough in the 460 nm region. As shown for several other kinetoplastids (Hill and Cross 1973; Hill 1976; Martin and Mukkada 1979), the α absorption maxima (555–558 nm) of cytochrome *c* are obscured by the relatively large amount of cytochrome *b* present in the organelle.

Preparation and spectral analysis of the mitochondrial red band fraction. A mitochondrial fraction was obtained from *L.*

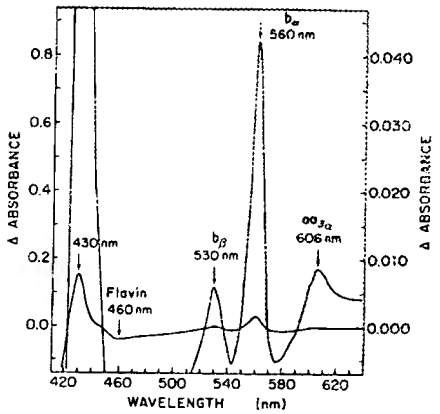

FIG. 1. Difference spectrum of *L. tarentolae* mitochondria at room temperature. The dithionite-reduced minus ferricyanide-oxidized difference spectrum of sonicated mitochondria was recorded in 0.25 *M* sucrose, 20 m*M* Tris-Cl, pH 7.9, and 2.0 m*M* EDTA on a scale of 1.0 for Δ absorbance (*y*-axis at left). The Δ absorbance for the amplified tracing is indicated on the *y*-axis at the right.

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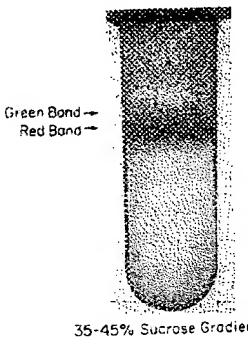

FIG. 2. (A) Mitochondria l
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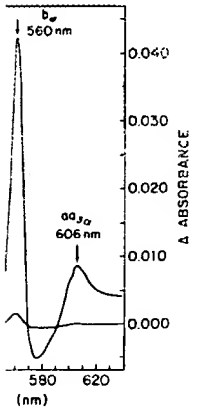

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tarentolae using the Renografin flotation method (Simpson and Braly 1970; Simpson and Simpson 1978) after hypotonic cell lysis. Sedimentation of a Triton-solubilized lysate through a 35–45% sucrose gradient yielded red and green protein bands (Fig. 2A). The Coomassie blue-stained profiles shown in Fig. 2B indicate that the red (lane 2) and green (lane 3) bands are enriched for different protein species, which represent complex subsets of the proteins present in whole mitochondria (lane 1).

Figure 3 shows the dithionite-reduced minus air-oxidized difference spectrum of the red band fraction. The α and β absorption maxima at 562 and 527 nm respectively, indicate that this fraction contains cytochrome *b*. After fractionation through the sucrose gradient, these bands were shifted 2–3 nm toward the red end of the spectrum. Spectral analysis at low temperature was used to show that multiple *b* cytochromes were not present in the red band. At 77K, the difference spectra revealed absorption maxima at 562 nm (α), 530 nm (β), and 422 nm (γ), again confirming that this fraction contained cytochrome *b* (unpublished results). In other organisms, nuclear-encoded cytochrome *b* was shown to produce two α peaks (557 and 550 nm) in a low temperature spectrum which was not detected in the red band fraction (Davis *et al.* 1972, 1973; von Jagow and Sebald 1980). The α absorption maxima of the *c* cytochrome(s) are obscured by the large amount of cytochrome *b* in this fraction, as indicated above in the spectral analysis of whole mitochondrial particles. A low level of the α absorption maximum for cytochrome aa_3 was also detected at 77K, suggesting that additional cytochromes may be present in this fraction. In fact, we have shown that the mitochondrial-encoded cytochrome oxidase subunit II polypeptide can be detected in this sample using a peptide antibody specific for the

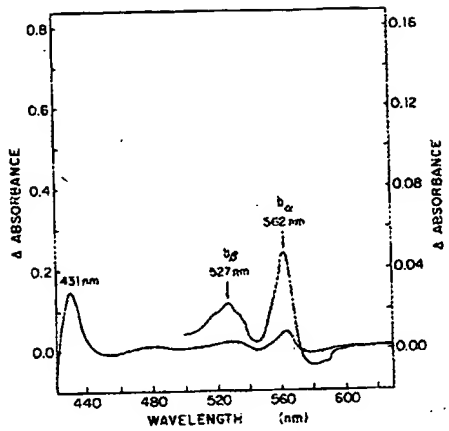

FIG. 3. Difference spectrum of the mitochondrial red band fraction at room temperature. The dithionite-reduced minus air-oxidized difference spectrum of the red band fraction was measured using a scale of 1.0 for Δ absorbance (y-axis at left). The red band sample in the solubilization buffer was collected directly from the gradient. An amplified tracing of the cytochrome *b* α and β peaks is also shown (absorbance scale at right).

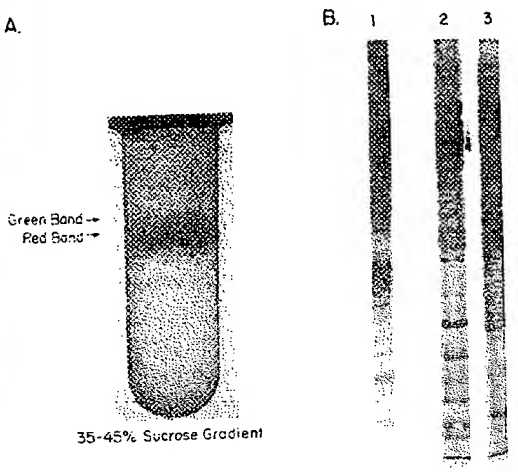

FIG. 2. (A) Mitochondria from *L. tarentolae* were solubilized in a buffer containing 2% Triton X-100 (see under Materials and Methods) and fractionated by band sedimentation through a 35–45% sucrose gradient. Two fractions (marked by arrows) were visualized as red and green bands in the gradient. (B) Coomassie blue-stained protein profiles of fractions separated on a 15% acrylamide/8 *M* urea/SDS gel: lane 1, total mitochondrial protein; lane 2, red band fraction; lane 3, green band fraction.

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carboxy terminus of the predicted protein (J. Shaw and L. Simpson, unpublished results).

Identification of cytochrome c_1 in the red band fraction. In other cells, the nuclear-encoded cytochrome c_1 is often released in a complex with cytochrome *b* when solubilization of mitochondria is performed using a nonionic detergent such as Triton X-100. The heme group of cytochrome c_1 is covalently linked to the apoprotein and is not separated from the polypeptide during electrophoresis on denaturing gels. To determine whether cytochrome c_1 is present in the red band fraction, the heme-associated peroxidase activity was assayed after denaturation and separation of proteins on a 10–20% SDS–polyacrylamide gel. When the gel was treated with TMBZ–H_2O_2, a 28-kDa protein from the red band fraction was strained (Fig. 4, TMBZ, lane 2, arrow), suggesting that this sample contained cytochrome *c*. The horse heart cytochrome *c* control was also stained very darkly (TMBZ, lane 1). Cytochrome *b* could not be identified after SDS gel electrophoresis because its heme group is not covalently linked with its polypeptide. Coomassie blue staining of the red band fraction after TMBZ–H_2O_2 staining identified a 28-kDa protein band (Fig. 4, Coomassie blue, lane 2, arrow) that comigrated with the 28-kDa heme peroxidase-labeled band. The molecular weight of this heme-binding protein is identical to that of bovine and yeast cytochrome c_1. Moreover, cytochrome *c*, which also contains a covalently bound heme group, is not present in this fraction, as shown by the lack of a 13-kDa heme-staining band.

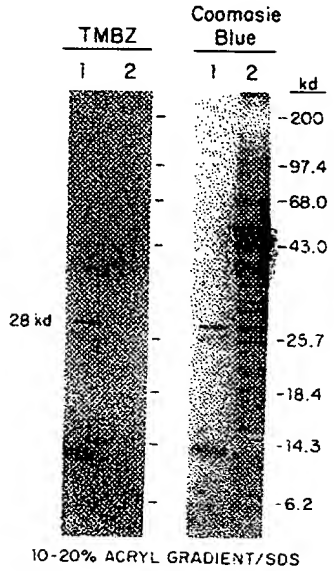

FIG. 4. TMBZ–H_2O_2 staining of a 28-kDa heme protein in the mitochondrial red band fraction. Protein samples were separated on a 10–20% acrylamide/SDS gradient gel and stained for heme-associated peroxidase activity with TMBZ–H_2O_2 (panel at left): lane 1, 0.01 μg horse heart cytochrome *c*; lane 2, 120 μg red band fraction. The arrow in lane 2 marks the 28-kDa heme protein identified as cytochrome c_1. The right panel shows the protein profiles of the same samples after staining with Coomassie blue. The arrow in lane 2 marks the 28-kDa heme protein identified by the heme staining in panel 1.

Amino acid analysis of the 28-kDa cytochrome c_1 protein. The amino acid composition of the 28-kDa heme-binding protein was determined after gel isolation, and was compared to the amino acid compositions of cytochrome c_1 for both bovine and yeast; the bovine composition is from the c_1 amino acid sequence (Wakabayashi *et al.* 1980) and the yeast data are derived from the gene sequence (Sadler *et al.* 1984), omitting the residues in the cleaved presequence. The results in Table 1 show that 14 of the 15 amino acid values determined for the *L. tarentolae* heme-binding protein are very similar to those obtained for the bovine and yeast cytochrome c_1 proteins. The high value for isoleucine is inconclusive since lower values (e.g., 11) were obtained in some hydrolysates and the higher value shown here may represent an artifact of this particular analysis (unpublished results). Cysteine and tryptophan, which are known to be labile under the hydrolysis conditions used, and proline, which was obscured by NH_3 in the sample, could not be determined.

Amino acid	28
Aspartic acid	
Glutamic acid	
Serine	
Glycine	
Histidine	
Arginine	
Threonine	
Alanine	
Proline	
Tyrosine	
Valine	
Methionine	
Isoleucine	
Leucine	
Phenylalanine	
Lysine	
Tryptophan	
Cysteine	
Total residues	
M_r	

[a] Not determined.

DISCUSSIO

The mitochondrial diff
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Cytochrome aa_3 is prese
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L. tarentolae cytochrome
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Solubilization of the *L*
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TABLE I
Amino Acid Composition Analysis

Amino acid	*L. tarentolae* 28-kDa heme protein	Bovine cytochrome c_1	Yeast cytochrome c_1
Aspartic acid	21	19	24
Glutamic acid	24	21	22
Serine	21	16	13
Glycine	25	17	18
Histidine	9	9	8
Arginine	15	15	14
Threonine	13	7	12
Alanine	19	18	24
Proline	—	22	22
Tyrosine	12	15	12
Valine	21	15	14
Methionine	4	10	5
Isoleucine	35	4	9
Leucine	15	25	18
Phenylalanine	10	8	10
Lysine	16	12	14
Tryptophan	ND[a]	3	5
Cysteine	ND[a]	5	4
Total residues		241	248
M_r	28 kDa	27.9 kDa	27.4 kDa

[a] Not determined.

Discussion

The mitochondrial difference spectrum reported here is characteristic of that found with other kinetoplastids (Hill and Cross 1973; Hill 1976; Martin and Mukkada 1979). Cytochrome aa_3 is present at a low level, while cytochrome *b* dominates the spectrum and completely masks the spectral contribution of the c_{555} cytochromes. The *L. tarentolae* cytochrome aa_3 α band has a maximum at 606 nm.

Solubilization of the *L. tarentolae* mitochondria in Triton X-100 followed by sucrose gradient centrifugation of the lysate yielded two fractions visualized as red and green bands (Fig. 2A). The red band contained cytochrome *b* as shown by spectral analysis of this fraction (Fig. 3). The composition of the green band was not investigated in this study, although preliminary results indicate the presence of flavoprotein (J. Shaw and L. Simpson, unpublished results). Coomassie blue-stained protein gel profiles (Fig. 2B) indicate the presence of a complex subset of mitochondrial proteins in the red band fraction.

Although our results indicated that absorption maxima for the nuclear-encoded *b* cytochromes were not present in the red band, we do not yet know whether the cytochrome *b* characterized in this fraction is encoded by the nuclear or mitochondrial genome of these cells. A transcriptionally active gene for cytochrome *b* has been found in the mitochondrial DNA of *L. tarentolae* but was shown to lack a 5′ AUG codon for the initiation of translation (de la Cruz *et al.* 1984). We recently showed that an AUG codon is created in the 5′ ends of the cytochrome *b* transcripts by an unusual form of RNA processing called RNA editing (Feagin *et al.* 1988; Shaw *et al.* 1988). An N-terminal amino acid sequence is required to determine the genomic origin of the mitochondrially localized cytochrome *b*

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protein and this data will also indicate whether or not edited mitochondrial cytochrome *b* RNAs are translated. The red band fraction described in this report should provide a starting point for the further purification of the cytochrome *b* protein.

There is a limited amount of indirect evidence in the literature suggesting that cytochrome c_1 is a component of the electron transport chain in kinetoplastids (Hill and White 1968; Kusel and Storey 1973). In this study, an absorption shoulder between 556 and 558 nm provided suggestive evidence that cytochrome c_1 was present in the red band fraction. A putative cytochrome c_1 protein was identified as a 28-kDa band in an SDS acrylamide gel which exhibited a heme-associated peroxidase activity. The amino acid composition analysis of this protein showed a strong similarity to the amino acid composition of cytochrome c_1 from both bovine and yeast. These results are consistent with the identification of the 28-kDa protein as cytochrome c_1 in *L. tarentolae*. It is likely, although not yet demonstrated, that cytochrome c_1 is present in a complex with cytochrome *b* as in other mitochondria.

Acknowledgments

This research was supported in part by a grant from the National Institutes of Health to L.S. (AI09102). J.M.S. was a predoctoral trainee on an NIH training grant (USPHS GM07104) and was also supported in part by a Mary Leonora Schulte Fellowship and an Ursula Mandel Fellowship. We thank Homero Dewes, Agda Simpson, and Gary Peters for helpful advice and stimulating discussions.

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inflammation around pulmonary blood vessels and airways. Moreover, high levels of Th2-associated cytokines (IL-4 and IL-5) were generated when lung-draining lymph node and tissue cells were restimulated with L. major %%%lysate%%%. These data suggest that the lung environment per se favors Th differentiation towards the Th2 phenotype.

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0011982821 BIOSIS NO.: 199900242481
Evaluation of recombinant K39 (rK39) antigen ELISA in the diagnosis of infantile visceral %%%Leishmaniasis%%% in South-West Saudi Arabia
AUTHOR: Ghalib H W (Reprint)
AUTHOR ADDRESS: Department of Clinical Microbiology and Parasitology, College of Medicine, King Saud University, Abha, Saudi Arabia**Saudi Arabia
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ABSTRACT: Recombinant K39 (rK39) and %%%Leishmania%%% donovani (Ld) %%%lysate%%% enzyme-linked immunosorbent assays (ELISA) detected high levels of anti-%%%Leishmania%%% specific IgG antibodies in infantile visceral %%%leishmaniasis%%% (VL) in Saudi Arabia. The mean optical density (OD) level of the anti-rK39 antibodies (2.113 +- 0.104) was significantly higher than the mean OD level of anti-Ld %%%lysate%%% antibodies (1.432 +- 0.082) ($p< 0.0001$). The sensitivity and specificity of rK39 and Ld %%%lysate%%% ELISA in detecting VL were 100% when comparing VL patients to normal endemic controls. rK39 ELISA was more specific than Ld %%%lysate%%% ELISA in identifying true VL from other coendemic infections like malaria and brucellosis (92.3%, 76.9%, respectively). rK39 antigen did not react with auto-reactive antibodies in autoimmune systemic lupus erythematosus (SLE) and was more specific than Ld %%%lysate%%% antigen in identifying anti-%%%Leishmania%%% specific antibodies from auto-reactive autoimmune antibodies. This suggests that rK39 ELISA has a good potential for sensitive and specific diagnosis of infantile VL in Saudi Arabia.

0011921676 BIOSIS NO.: 199900181336
Immunoglobulin subclass distribution and diagnostic value of %%%Leishmania%%% donovani antigen-specific immunoglobulin G3 in Indian kala-azar patients
AUTHOR: Anam Khairul; Afrin Farhat; Banerjee Dwijadas; Pramanik Netai; Guha Subhasis K; Goswami Rama P; Gupta Pratap N; Saha Shiben K; Ali Nahid (Reprint)
AUTHOR ADDRESS: Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Rd., Calcutta, 700032, India**India
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0010264435 BIOSIS NO.: 199698732268
RK39: A cloned antigen of %%%Leishmania%%% chagasi that predicts active

Biomed Res 1999; 10 (1): 1-7

EVALUATION OF RECOMBINANT K39 (RK39) ANTIGEN ELISA IN THE DIAGNOSIS OF INFANTILE VISCERAL *LEISHMANIASIS* IN SOUTH-WEST SAUDI ARABIA

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Key words: Visceral leishmanisis, ELISA, rK39

ABSTRACT

Recombinant K39 (rK39) and *Leishmania donovani* (Ld) lysate enzyme-linked immunosorbent assays (ELISA) detected high levels of anti-*Leishmania* specific IgG antibodies in infantile visceral leishmaniasis (VL) in Saudi Arabia. The mean optical density (OD) level of the anti-rK39 antibodies (2.113 ± 0.104) was significantly higher than the mean OD level of anti-Ld lysate antibodies (1.432 ± 0.082) ($p < 0.0001$). The sensitivity and specificity of rK39 and Ld lysate ELISA in detecting VL were 100% when comparing VL patients to normal endemic controls. rK39 ELISA was more specific than Ld lysate ELISA in identifying true VL from other coendemic infections like malaria and brucellosis (92.3%, 76.9%, respectively). rK39 antigen did not react with auto-reactive antibodies in autoimmune systemic lupus erythematosus (SLE) and was more specific than Ld lysate antigen in identifying anti-*Leishmania* specific antibodies from auto-reactive autoimmune antibodies. This suggests that rK39 ELISA has a good potential for sensitive and specific diagnosis of infantile VL in Saudi Arabia.

INTRODUCTION

Visceral leishmaniasis (kala-azar) is endemic in the south-west of Saudi Arabia [1; 2] and is a major health problem in the region [3]. The disease resembles the Mediterranean type of infantile kala-azar affecting mainly infants and young children and is usually associated with severe morbidity and high mortality in untreated cases [4; 5]. It is zoonotic in nature [6; 7], caused by *Leishmania donovani sensu*

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lato zymodeme LON 42 [8; 3; 6] and transmitted by Phlebtomine flies in the low lands of Southwestern Saudi Arabia "Tihama region".

Prompt diagnosis, management and treatment of suspected cases are essential to reduce the severe morbidity and high mortality associated with active visceral leishmaniasis (kala-azar). Such critical interventions are hampered by the lack of sensitive and specific diagnostic methods. Definitive diagnosis of visceral leishmaniasis, still relies on invasive procedures for the detection of parasite amastigotes in aspirates and/or biopsies of lymph nodes, bone marrow or spleens. These procedures are laborious, invasive and lack sensitivity [9].

Visceral leishmaniasis is invariably associated with high titers of anti-*Leishmania* specific IgG class antibodies and as such a battery of diagnostic serologic tests were developed as alternatives to parasite detection [10; 11; 12]. The sensitivity and specificity of such diagnostic methods depend on the type, source, and purity of antigen employed [13; 14]. The introduction of purified or cloned proteins instead of whole crude parasite antigens in the serologic tests greatly increased the sensitivity and specificity of these tests. Of these cloned proteins is recombinant K39 (rK39), a 39 amino acid repeats encoded by a kinesin-like gene of *Leishmania chagasi* amastigotes [15]. rK39 antigen was found to be sensitive and specific for the serological detection of visceral Leishmaniasis in many of the endemic parts of the world including Sudan, Brazil, China and India and Southern Europe [15; 16; 17; 18].

In the present study we evaluated the potential and validity of rK39 antigen based enzyme-linked immunosorbent assay (ELISA) in the diagnosis of infantile visceral leishmaniasis in the south-west of Saudi Arabia.

MATERIALS AND METHODS

Patients and controls:

Infantile Visceral leishmaniasis (VL) patients

The VL patients included in this study were from the endemic Tihama region of Asir lowlands in the South-west Saudi Arabia. These were referred to Asir Central Hospital (the study site) for definitive diagnosis and treatment. Suspected patients had fever of more than two weeks duration, splenomegaly, weight loss and pancytopenia. On admission most of the patients were severely ill, some required supportive management with fluids, antibiotics and few others required blood transfusions. All VL suspected patients had bone marrow aspirations for the detection of *Leishmania* parasite amastigotes. These patients were negative for the other coendemic diseases such as malaria, brucellosis and typhoid fever with similar clinical features of fever, weight loss and hepatosplenomegaly.

28 VL patients were selected and included in the serodiagnostic study. Of these, 22 out of the 28 were bone marrow positive for *Leishmania* amastigotes; and the other 6 were bone marrow negative for *Leishmania* parasite amastigotes but responded dramatically to anti-leishmanial therapy.

The average age of the patients was less than 2 years and only one patient was 4 years old. They responded quite well to sodium stibogluconate 10 mg/kg/day for 16-21days and did not require a second course for failure of treatment or clinical relapse.

Control groups:

Malaria and brucellosis

8 malaria and 5 brucella patients admitted to the paediatric ward, Asir Central Hospital were included in the study.

Autoimmune patients

9 patients with autoimmune systemic lupus erythematosus (SLE) were included in the study.

Normal controls

16 normal blood donors who were negative for HIV and viral hepatitis, with no previous history of either visceral or cutaneous leishmaniasis were included in the study as normal endemic controls.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples collected from all patients and controls were tested for anti-*Leishmania* specific IgG antibodies by ELISA. The recombinant K39 (rK39) was prepared as we described earlier [15]. The soluble *L. donovani* lysate (Ld lysate) used for comparison with rK39 was prepared as we previously described [11].

The samples were tested for their reactivity with the antigens following standard ELISA methods that we described elsewhere [11; 15]. In brief, Corning polystyrene microtiter plates were coated overnight at 4 C with 50 ng of rK39 or 1µg Ld lysate per well in 50 µl of 0.1 bicarbonate buffer, pH 9.0. The wells were then blocked with 200 µl of 1% bovine serum albumin for 2 hr, washed 4 times with PBS (phosphate-buffered saline, pH 7.4)-Tween 20 (1%) and incubated at 37 °C for 30 min with 50 µl of patients or control sera diluted 1:50 in PBS-Tween (0.1%). The wells were again washed 4 times with the same wash buffer and incubated with 50 µl of goat anti-human igG (Fe specific) peroxidase conjugate (Sigma Chemical Co., St. Louis, Missouri, USA) at a dilution of 1:60,000. After incubation for 30 min at room temperature, the wells were washed again and incu-

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bated with 100 µl of TMB microwell peroxidase substrate (Kirkegaard & Perry Labs., Gaithersburg, MD, USA) for 20-30 min, the reaction was stopped with 100 µl 1 N H2SO4. The optical density was read at 450 nm in a plate reader (ICN Titertek Multiscan MCC/340, Labsystems, Finland). Each sample was assayed in duplicate or more, together with the appropriate positive and negative serum controls. The ELISA reader was set to subtract the reading of a blank control from the test samples. A cut off value above which the sample was considered positive was set at an OD reading 3 standard deviations above the mean of the normal.

Data and statistical analysis

The data was entered, graphed and analysed using GraphPad Prism Version 2.00 (GraphPad Software, Inc., San Diego, California, USA).

RESULTS

Comparison of rK39 and Ld lysate ELISA in the detection of Infantile visceral leishmaniasis

Both rk39 and Ld lysate ELISA assays detected high levels of anti-*Leishmania* specific IgG antibodies in all the VL patients' sera and none of the normal control sera reacted to the antigens. The mean optical density (OD) level of the anti-rk39 antibodies (2.113 ± 0.104) was significantly higher than the mean OD level of anti-Ld lysate antibodies (1.432 ± 0.082) ($p< 0.0001$). The sensitivity and specificity of the ELISA assay utilising either of the two antigens in detecting VL were 100%, when comparing the 28 VL patients to the 16 normal endemic controls (Fig 1).

We tested the sensitivity and specificity of the ELISA assay in differentiating true VL from malaria and brucellosis. The ELISA assay using rK39 was positive in 1 out of 8 malaria patients' sera and none of the brucella patients reacted to rK39, while the ELISA assay using Ld lysate was positive in 1 out 8 malaria patients and in 2 out of 5 brucella patients (Fig 2). The sensitivity of the ELISA test using rK39 was 100% and the specificity was 92.3%, while the sensitivity of the test using Ld lysate was 100% and the specificity was 76.9%.

Since autoimmune diseases may sometimes induce auto-reactive antibodies which may give false positive serologic tests, we tested the ELISA assays comparing both antigens for the detection of none specific antibodies in SLE patients. rK39 ELISA was negative in all the SLE patients' sera, while Ld lysate ELISA assay was positive in 1out of 9 SLE patients (Fig 3).

DISCUSSION

Infantile visceral leishmaniasis is a major health problem in the south -west of Saudi Arabia and is associated with severe morbidity and high mortality in untreated cases [3; 4; 5]. Clinical diagnosis is complicated by the prevalence of other coendemic infections with similar clinical manifestations like malaria and brucellosis. Still, the definitive diagnosis of VL in this region relies on non-sensitive, invasive procedures for the detection of parasite amastigotes in bone marrow aspirates [4]. The bone marrow procedure was less than 60% sensitive in detecting VL among our group of patients and as reported elsewhere [19]. At the same time, the splenic aspiration procedure which is more sensitive than the bone marrow for the detection of parasites is not practiced in the region because of reluctance in patient's compliance and fear of associated complications in patients with severe morbidity. As such, serodiagnosis remains as a favourable safe, sensitive and specific choice in the diagnosis of VL.

The present study is an extension of the previous rK39 studies [15; 16;17; 18] demonstrating its potential as a sensitive and a specific antigen in ELISA for the serodiagnosis of infantile visceral leishmaniasis in Saudi Arabia. Both rK39 and Ld lysate antigen ELISA assays had similar high power in the diagnosis of visceral leishmaniasis in patients with high clinical suspicion in infants and young children in whom other similar coendemic infections were excluded (Fig 1).

rK39 antigen ELISA was more specific than Ld lysate ELISA in identifying true VL from other coendemic infections (92.3%, 76.9%, respectively) (Fig 2). The malaria patient who was positive in rK39 ELISA probably came from a *Leishmania* endemic

Fig. 1: rK39 and Ld lysate ELISA assay for anti-*Leishmania* specific IgG antibodies in the VL patients and normal endemic control groups: OD values of anti-rK39 (closed circles) and anti-Ld lysate (open circles) specific IgG antibodies in visceral leishmaniasis (VL) and normal endemic control (Normal) group are expressed in a scattergram. The solid horizontal bars represent the mean OD value for each group. The hatched horizontal bars represent the cut off value for each antigen as described in the text.

Fig. 2: rK39 and Ld lysate ELISA comparing the reactivity of VL, malaria, brucella patients and normal endemic control group (Normal) to rK39 and Ld lysate antigens: The OD reactivity of each group sera are shown in a scattergram. Anti-rK39 OD values are shown in closed circles and anti-Ld lysate OD values in open circles. Mean OD values for each group are represented with solid horizontal bars. The hatched horizontal bars represent the cut off value for each antigen as described in the text.

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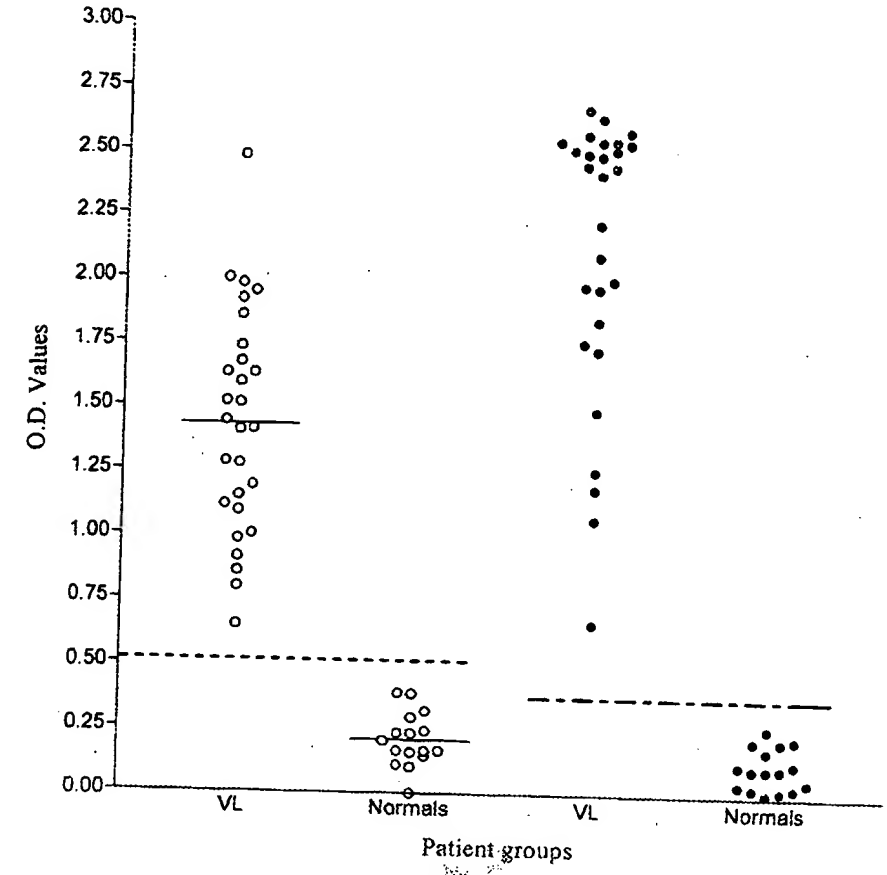

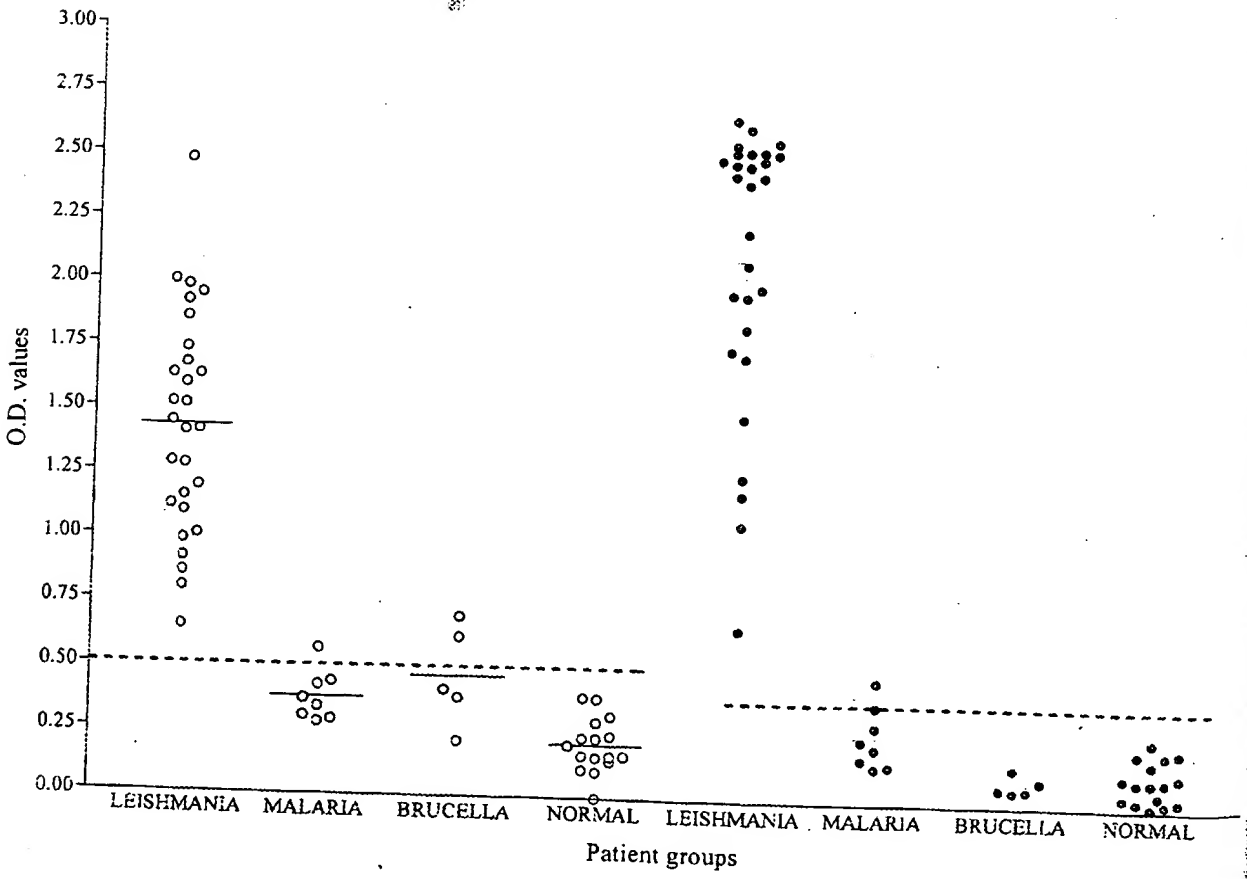

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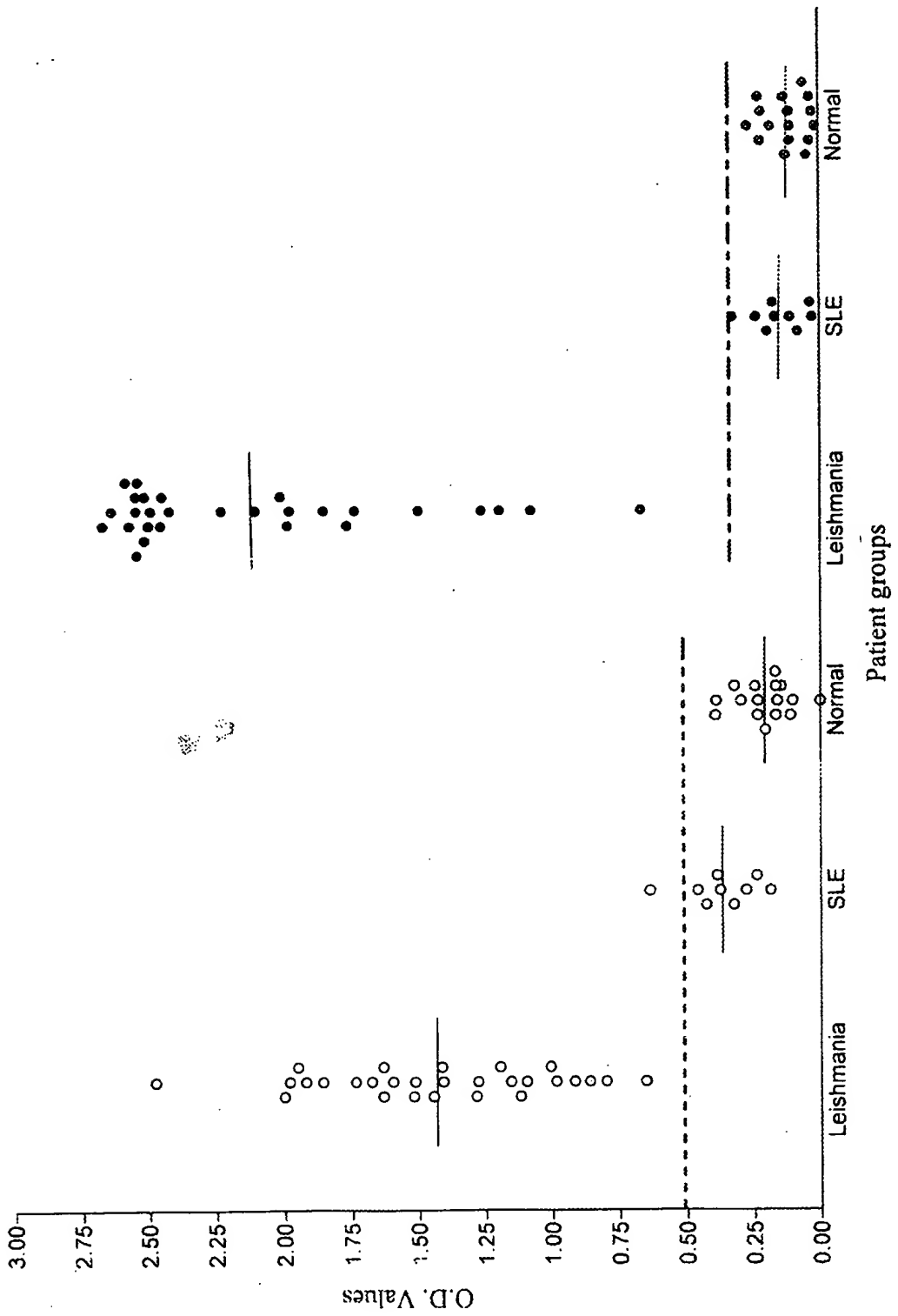

Fig. 3: ELISA assay comparing the VL patients', SLE patients' and Normal control group sera reactivity to rK39 and Ld lysate antigens: The OD reactivity of each group sera are shown in a scattergram. Anti-rK39 OD values are shown in closed circles and anti-Ld lysate OD values in open circles. Mean OD values for each group are represented with solid horizontal bars. The hatched horizontal bars represent the cut off value for each antigen as described in the text

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focus and had previous visceral or cutaneous leishmaniasis. In similar studies in the Sudan we found that anti-rK39 antibodies could be positive in subclinical, active or past infections [20]. In contrast, anti-rK39 antibodies in Brazilian VL were found to be only associated with active disease and were absent in children who have subclinical disease and self-heal without specific therapy [16]. However the clinical diseases in Sudan and Brazil are different. Brazilian VL is more of an infantile disease while the diseases in Sudan affects all ages, is more severe and occurs in cyclic epidemic episodes. The exact association of reactivity to rK39 antigen need to be determined in the case of infantile VL in Saudi Arabia. This requires a long term longitudinal study in an endemic foci in the region. If rK39 reactivity is found to be only associated with active disease, then the rK39 diagnostic ELISA will be more specific and will have a great prognostic value similar to the case in Indian VL [18]. In our experience in the last two years, most of the highly suspected infantile VL patients who were bone marrow negative but were positive on rK39 ELISA responded to specific VL treatment. The nature of the rK39 antigen and its long term stability, the feasibility of preparing large amounts of cloned antigen make it favourable for the development of standard simple diagnostic kits for the diagnosis of VL.

Since autoimmune diseases may sometimes induce auto-reactive antibodies which may give false positive serologic reactivity, we tested the ELISA assays comparing both antigens for possible non-specific reactivity with auto-reactive antibodies in SLE patients. rK39 ELISA was more specific than Ld lysate antigen in differentiating between anti-leishmania specific antibodies and non-specific auto-reactive antibodies in SLE patients (100% and 88.9% respectively) (Fig 3).

The current results thus suggests that rK39 ELISA has a great potential for sensitive and specific diagnosis of infantile VL in Saudi Arabia. Conclusive evidence for this and the prognostic value of rK39 ELISA awaits further investigation using a large sample longitudinal study.

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AUTHOR: Stiteler J M (Reprint); Grogl M; Rowton E D
AUTHOR ADDRESS: Department of Entomology, Division of Communicable Diseases and Immunology, Walter Reed Army Institute of Research, Washington, DC, USA**USA
JOURNAL: American Journal of Tropical Medicine and Hygiene 62 (3 Supplement): p310 March, 2000 %%%2000%%%
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0012449530 BIOSIS NO.: 200000167843
Site of antigen delivery can influence T cell priming: Pulmonary environment promotes preferential Th2-type differentiation
AUTHOR: Constant Stephanie L (Reprint); Lee Karen S; Bottomly Kim
AUTHOR ADDRESS: Section of Immunobiology/LH404, Yale University School of Medicine, New Haven, CT, 06520-8011, USA**USA
JOURNAL: European Journal of Immunology 30 (3): p840-847 March, 2000 %%%2000%%%
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ISSN: 0014-2980
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ABSTRACT: Delivery of foreign antigens to mucosal surfaces, such as the pulmonary airways, has been shown to preferentially induce Th2-mediated responses in humans and in mice. What is not clear from these studies is whether this preferential skewing in responses is the result of the limited types of antigen being administered and/or a bias towards using particular genetic strains of mice, or whether the lung environment in itself provides a favored site for the priming of Th2-type cells. We have addressed this issue using an antigen/mouse strain combination that, under typical conditions of immunization, is strongly biased towards priming for TH1 CD4+ T cells. We show that %%%Leishmania%%% major parasites deliverated to C57BL/6 mice via an intranasal route fail to induce the expected Th1-dominated responses and instead preferentially prime for Th2 responses. These included an influx in lymphocytes and eosinophils into alveoli, as well as the induction of Th2-type foci of

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411 ODS LEISHMANIA SKIN TEST, MFL-LSTA[R2]: STABILITY OF THE CGMP PRODUCT IN THE GUINEA PIG ANIMAL MODEL. Stiteler, JM, Grogl, M, and Rowton, ED. Department of Entomology, Division of Communicable Diseases & Immunology, Walter Reed Army Institute of Research Washngton, DC; and Dept of Biologics Research, Division of Communicable Diseases & Immunology, Walter Reed Army Institute of Research Washngton, DC.

The Operation Desert Storm (ODS), heat-treated, microfluidized, lysate (MFL), Leishmania skin test (LSTA) referred to as MFL-LSTA[R2], which was developed and produced at WRAIR, has been undergoing Stability Testing. MFL-LSTA[R2] was produced in accordance with Food & Drug Administration (FDA) guidelines for current Good Manufacturing Practices (cGMP) within WRAIR's Pilot Bioproduction Facility in June 1998 (Lot No. 0564), at which time a Stability Testing Protocol in the LSTA Guinea Pig Animal Model was initiated. The Delayed-Type Hypersensitivity (DTH) results in the ODS strain (*Leishmania tropica* strain WR#1063) indicated that the MFL-LSTA[R2] product Lot No. 0564 (from normal storage conditions at 4 C) has remained efficacious in Guinea Pigs sensitized with the ODS strain for a period of 2 years. The Investigational New Drug Application (INDA) submitted for review to FDA, resulted in Release of Lot No. 0564 for Clinical Trials. The Phase 1 Clinical (Safety) Trial was completed successfully at WRAIR in 1999. Phase 2a Efficacy Trials are being planned in up to three overseas field sites including both the Old World as well as the New World.

412 CHARACTERIZATION OF STRAIN OF *TRYPANOSOMA CRUZI* FROM *DIDELPHIS MARSUPIALIS* OF URBAN AND RURAL AREAS. Lugo de Yarbuh A, Moreno E, Gonzalez N, Rivera I, Guillen B, Alarcon M, Payares G, and Colasante C. Investigaciones Parasitologicas *J F Torrealba*, Facultad de Ciencias, Universidad de los Andes; Sección de Parasitología, Facultad de Ciencias, Universidad Central de Venezuela; and Laboratorio de Fisiología de la Conducta, Facultad de Medicina, Univesidad de los Andes.

Didelphis marsupialis is a primary reservoir of *Trypanosoma cruzi* highly adapted to live in urbanized envionments (UE). We are investigating strains of *T. cruzi* isolated from these animals and those in rural areas (RA), where Chagas' disease is endemic. Peripheral blood from 40 *D. marsupialis* (30 RA and 10 UE) was examined in Giemsa-stained thin smears to detect bloodform trypomastigotes. For xenodiagnosis 10 third-stage nymphs of *Rhodnius prolixus* were engorged on each oposssum. At four week after the bugs were fed, the urine was collected. Blood samples from the opossum and urine samples from the bugs containing metacyclic forms were intraperitoneally inoculated into NMRI mice. Sections of different mouse organs were stained with haematoxylin-Eosin and examined to detect parasite nests and tissue alterations. Each mouse was examined to determine patent parasitemia and mortality. Five strains *T. cruzi* (UE) and 20 strains from RA were isolated in hemoculture NNN medium, and used for isoenzyme (PGM, GPI, IDH-NADP, ME, MDH, G6PDH, MPI and 6PHDH) and k-DNA-pattern determination. PCR assay was used with primers: F1 (5'-GTCGGAGCAGGGACAGC-3') and F2 (5'-ACATCTGGAACCTCTCCC-3'). The results showed differences between *T. cruzi* parasites in the two groups: in clinical forms, virulence, tissue tropism and pathology. *T. cruzi* from RA caused megaviscera, skeletal muscle fiber alterations, heart and brain lesions, leading to death during the acute phase. *T. cruzi* from UE showed lower parasitemia, reaching the chronic phase. Five isoenzyme patterns were different in one *T. cruzi* strains from RA (PGM, IDH-NADP, MDH, MPI, 6PGDH), indicating that *T. cruzi* strains are heterogeneous even when isolated from the same host. All the isolates showed a single specific *T. cruzi* band at 110, 125 or 130 bp of amplification, suggesting the presence of three *T. cruzi* subpopulations in the same host species.

413 PREVALENCE OF ANTIBODY TO *TRYPANOSOMA CRUZI* IN THE TOLEDO DISTRICT OF BELIZE. Southern PM, Walker KP, Race EMR, and Patel SJ. Departments of Pathology and Internal Medicine (Inf. Dis.), University of Texas Southwestern Medical Center, Dallas, TX; and Chief Cornerstone, Punta Gorda, Belize.

Infections due to *Trypanosoma cruzi* (Tc) are endemic from Central America southward to some of the southern cone countries of South America. The prevalence of Tc infection in Belize has not been studied systematically for many years. We enrolled persons from 27 towns or villages in the Toledo District of Belize for this study. After obtaining consent, we collected finger-prick blood onto filter paper. The age, sex, ethnic group and village were recorded. The samples were tested for antibody (Ab) to Tc by an EIA method, with positive/negative controls (including known leishmania infection). Of a total of 500 subjects tested,

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%%%leishmanial%%% antigen that elicits IL-12 production and Th1-type responses in patients as well as IL-12 production in normal human PBMC.

0009719392 BIOSIS NO.: 199598187225
Cytotoxicity in human mucosal and cutaneous %%%leishmaniasis%%%
AUTHOR: Barral-Netto M (Reprint); Barral Aldina; Brodskyn Claudia; Carvalho E M; Reed S G
AUTHOR ADDRESS: Serv. Imunologia-HUPES-UFBA, R. Joao das Botas s/n, 40110-040 Salvador, Bahia, Brazil**Brazil
JOURNAL: Parasite Immunology (Oxford) 17 (1): p21-28 1995 %%%1995%%%
ISSN: 0141-9838
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: CD8+ T cells and lysis of parasitized macrophages seem to be important in the resistance to murine %%%leishmaniasis%%%. In the present study, we evaluated peripheral blood mononuclear cell (PBMC) from patients with either cutaneous (CL) or mucosal (ML) %%%leishmaniasis%%% in cell lysis assays using 51-Cr-labeled Daudi or K562 cells, or autologous antigen-pulsed macrophages as targets. Results are reported as lytic units (number of cells required for 30% lysis) per million PBMC. Exposure of patient PBMC (n = 12) to %%%lysate%%% from %%%Leishmania%%% amazonensis promastigotes led to an increase in cytotoxic activity compared to unstimulated patient cells against Daudi (81.8 +- 14.9 vs 13.6 +- 5 lytic units (LU) per million PBMC; mean +- SEM) and K562 (65.7 +- 8.4 vs 13.1 +- 5 LU/10^-6 PBMC). ML had higher responses when CL in

0008772404 BIOSIS NO.: 199395074670
Mapping human T cell epitopes in %%%Leishmania%%% gp63: Identification of cross-reactive and species-specific epitopes
AUTHOR: Russo Donna M; Jardim Armando; Carvalho Edgar M; Sleath Paul R; Armitage Richard J; Olafson Robert W; Reed Steven G (Reprint)
AUTHOR ADDRESS: Seattle Biomed. Research Inst., 4 Nickerson St., Seattle, WA 98109, USA**USA
JOURNAL: Journal of Immunology 150 (3): p932-939 %%%1993%%%
ISSN: 0022-1767
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

0008292116 BIOSIS NO.: 199293135007
A RIBONUCLEASE ACTIVITY IS ACTIVATED BY HEPARIN OR BY DIGESTION WITH PROTEINASE K IN MITOCHONDRIAL EXTRACTS OF %%%LEISHMANIA%%%-TARENTOLAE
AUTHOR: SIMPSON A M (Reprint); BAKALARA N; SIMPSON L
AUTHOR ADDRESS: DEP BIOLOGY MOLECULAR BIOLOGY INSTITUTE, UNIVERSITY CALIFORNIA, LOS ANGELES, CALIF 90024, USA**USA
JOURNAL: Journal of Biological Chemistry 267 (10): p6782-6788 %%%1992%%%
ISSN: 0021-9258
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: A ribonuclease activity in a 100,000 .times. g supernatant of a Triton %%%lysate%%% of a mitochondrial-kinetoplast fraction from %%%Leishmania%%% tarentolae is activated by incubation with heparin or by predigestion of the %%%lysate%%% with proteinase k or pronase. In vitro-transcribed preedited cytochrome b mRNA is cleaved at several

Parasite Immunology, 1995: **17**: 21–28

Cytotoxicity in human mucosal and cutaneous leishmaniasis

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SUMMARY

$CD8^+$ T cells and lysis of parasitized macrophages seem to be important in the resistance to murine leishmaniasis. In the present study, we evaluated peripheral blood mononuclear cell (PBMC) from patients with either cutaneous (CL) or mucosal (ML) leishmaniasis in cell lysis assays using ^{51}-Cr-labeled Daudi or K562 cells, or autologous antigen-pulsed macrophages as targets. Results are reported as lytic units (number of cells required for 30% lysis) per million PBMC. Exposure of patient PBMC (n = 12) to lysate from Leishmania amazonensis *promastigotes led to an increase in cytotoxic activity compared to unstimulated patient cells against Daudi ($81{\cdot}8 \pm 14{\cdot}9$ vs $13{\cdot}6 \pm 5$ lytic units (LU) per million PBMC; mean ± SEM) and K562 ($65{\cdot}7 \pm 8{\cdot}4$ vs $13{\cdot}1 \pm 5$ LU/10^6 PBMC). ML had higher responses than CL in both targets ($80{\cdot}4 \pm 11{\cdot}0$ vs $46{\cdot}4 \pm 11{\cdot}6$ LU/10^6 PBMC for K562, and $104{\cdot}3 \pm 23{\cdot}8$ vs $59{\cdot}3 \pm 14{\cdot}3$ LU/10^6 PBMC for Daudi). Normal control PBMC, stimulated with* L. amazonensis *antigen had $6{\cdot}32 \pm 3{\cdot}72$ LU/10^6 PBMC against Daudi cells and $9{\cdot}06 \pm 2{\cdot}78$ LU/10^6 PBMC against K562. The cell responsible for lysis of the K562 cells was characterized as NK, by means of cell separation employing magnetic beads coupled to antibodies. Addition of recombinant TGF-β or recombinant human IL-10 reduced* L. amazonensis*-induced cytotoxicity by 90% and 70%, respectively. Cytotoxicity of antigen-stimulated PBMC was also demonstrated against autologous* L. amazonensis *antigen-pulsed macrophages in the range of 6·7 to 41·7 LU/10^6 PBMC. In this system TGF-β and IL-10 also decreased the antigen-induced cytotoxic response.*

Keywords *leishmania, cytotoxicity, immunity*

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INTRODUCTION

Leishmaniasis is a major public health problem in several parts of the world. Once inside the mammalian host, leishmania are obligatory intra-macrophage parasites, multiplying in these cells as non-motile amastigotes. In all forms of leishmaniasis recovery from and resistance to disease are dependent on protective T cell responses (Russo *et al.* 1993).

In the response against intracellular pathogens T lymphocytes act by two distinct mechanisms: either through the activation of the host cell leading to intracellular killing of the pathogen or by its capacity of lysing infected target cells. These two mechanisms are related to antigen presentation and are mediated by two phenotypically distinct T lymphocyte subpopulations. $CD8^+$ positive T lymphocytes are specific for antigen in the context of Class I MHC molecules. Such characteristics are believed to be related to antiviral responses. The response against intra-macrophage parasites as Leishmania has been traditionally believed to be related to lymphokine production by $CD4^+$ T cells.

Recent evidence suggests an important role for cytotoxic $CD8^+$ T cells in the response against intracellular protozoa. In malaria cytotoxic T cells are of importance in the immunity against sporozoites (Weiss *et al.* 1988, Kumar *et al.* 1989). Such cells are also important in the response against *Listeria monocytogenes* (De Libero & Kaufmann 1986), and *Mycobacterium leprae* (Stach *et al.* 1982, Kaplan *et al.* 1988, Modlin *et al.* 1988, Hancock, Cohn & Kaplan 1989).

Although most of the studies in leishmaniasis indicate a role in protective immunity for IFN-γ producing $CD4^+$ Th1 helper T cells (Liew, Hale & Howard 1982, Milon *et al.* 1986, Farrell, Muller & Louis 1989) there are indications for the participation of cytotoxic T cells. $CD8^+$ T cells are able to mediate protective immunity in murine leishmaniasis following elimination of $CD4^+$ T cells in BALB/c animals (Hill, Awwad & North 1989), and in human cutaneous leishmaniasis healing is associated with increased numbers of leishmania-specific $CD8^+$

cells. This report investigates the presence of cytotoxic cells in the peripheral blood of patients with different forms of tegumentary leishmaniasis and the regulation of such activity by cytokines.

MATERIAL AND METHODS

Patient selection and characterization

Participants of the present study include six patients with cutaneous leishmaniasis and six patients with mucosal leishmaniasis from the endemic area of tegumentary leishmaniasis in Corte de Pedra, Bahia State, Brazil. All patients had a classical clinical picture of cutaneous or mucosal leishmaniasis, and the clinical diagnosis was confirmed by at least one laboratory test (serology, skin-test or parasite culture). A detailed evaluation including a complete physical examination was performed on each patient. Anti-leishmania antibodies were detected by a standard alkaline-phosphatase ELISA procedure as described elsewhere (Badaro *et al.* 1986). Results are expressed as OD_{402nm} measurements ($\times 1000$) at a 1/100 serum dilution. The reaction was considered positive when the OD was above 0·05. Montenegro's skin reaction was performed using 20 μg protein of crude leishmanial antigen as previously described (Reed *et al.* 1986). After 48 h the largest diameter of the indurated area was measured and considered positive if >5 mm. The aspirated material from tegumentary ulceration was aseptically inoculated directly into tubes of NNN blood agar overlaid with a modified liver infusion tryptose (LIT) medium and 10% heat-inactivated fetal calf serum (hiFCS) (Barral *et al.* 1987). Cultures were kept at 25°C for up to two months and checked weekly for parasite growth. Positive cultures were transferred to modified LIT with 10% hiFCS and incubated at 25°C. Stocks were stored in liquid nitrogen (−190°C) using 7·5% dimethylsulphoxide (DMSO) in LIT medium containing 30% hiFCS. Positive leishmania cultures were prepared for characterization during the two initial passages by procedures already described (Grimaldi, David & McMahon-Pratt 1987). Serodome analysis was performed using panels of anti-leishmania monoclonal antibodies (MoAbs) specific for members of the *Leishmania braziliensis*, *L. mexicana* and *L. donovani* complexes already described (McMahon-Pratt, Bennett & David 1982, Jaffe *et al.* 1984, Grimaldi, David & McMahon-Pratt 1987) in an indirect radioimmune binding assay (Grimaldi, David & McMahon-Pratt 1987). All patients received conventional antimonial (Glucantime) treatment consisting of Sb^v 20 mg/kg/day for 20 days. Informed consent was obtained from all patients, and the study was approved by the Committee on Human Rights of the Hospital Universitario Edgard Santos (University of Bahia).

Reagents

All cytokines except for murine recombinant IL-10 (a gift from Dr Robert Coffman, DNAX, Palo Alto, CA, USA and used at 100 U/ml) were human recombinant products. TGF-β (a gift from Dr D.Twardzik, Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA, USA) was used at 2 or 10 ng/ml; IL-10 (a gift from Dr Robert Coffman, DNAX) was used at 10 or 40 U/ml; IFN-γ (a gift from Dr Maud Brandely, Institute Roussell Uclaf, Paris, France) was used at 100 or 200 IU/ml; IL-2 or IL-4 (both from Immunex Corporation, Seattle, WA, USA) were used at 10 U/ml and 10 ng/ml respectively.

Anti-TGF-β MoAb (1D11.16) a gift from Dr L. Ellingsworth (Celtrix Laboratories Inc, Palo Alto, CA, USA), was used at 1 or 20 μg/ml. Anti-human IL-10 (Dr Robert Coffman, DNAX) was used at 20 μg/ml. Anti-IFN-γ was used at 1 μg/ml. Parasite (*L. amazonensis*; BA-32 strain) culture for antigen preparation was performed in modified liver infusion tryptose medium supplemented with 10% fetal calf serum. The parasite antigen was used at 10 μg/ml and consisted of freeze-thawed promastigotes centrifuged at 10 000 *g* for 30 min and filtered through a 0·22 μm filter.

Effector cells

Patient PBMC were obtained from heparinized venous blood. Cells were separated over Ficoll-Hypaque gradient and used fresh or kept frozen in liquid nitrogen until use in the assays. Cells were washed and resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated pooled human AB serum, at a concentration of 2×10^6 cells/ml and stimulated with leishmania antigen for four days in a humid atmosphere with 5% CO_2.

Tumour target cells

Target cells were prepared from suspension cultures of the tumour cell lines K562 (NK susceptible) and Daudi (NK resistant) (American Type Culture Collection, Rockville, MD, USA) maintained in RPMI 1640 with 10% fetal calf serum. Target cells were radiolabelled by suspension at a concentration of 10^7 cells/ml in medium containing 200 mCi/ml of 51-Cr sodium chromate for

90 min at 37°C, washed thrice and resuspended in medium at a concentration of 4×10^4 cells/ml.

Autologous target cells

PBMC were plated at $1{\cdot}5 \times 10^6$ cells/well in round-bottomed microtitre plates in order to obtain adherent cells. After two hours non-adherent cells were washed off and fresh complete medium was added. After five days plates were further washed and wells received leishmania antigen (10 μg/ml) or maintained without antigen. On day six wells were washed again and received two mCi/well of 51-Cr sodium chromate for 18 h. After further washings cells were used in cytotoxicity assays.

Cytotoxicity assay

Tumour radiolabelled target cells at 2×10^3 cells/well were dispensed in 50 ml aliquots into wells of round-bottomed microtitre plates (Nunc) and incubated in RPMI 1640 medium with 10% FCS with different numbers of effector cells (ranging from 3:1 to 100:1 effector:target ratio) for 4 h at 37°C in a humid atmosphere with 5% CO_2. In autologous target cell assay, effector cells were added at different numbers with radiolabelled target cells in round-bottomed microtitre plates. Cells were incubated again for 18 h to allow for target cell lysis. Supernatants were harvested (Skatron Harvesting System, Norway) and the radioactivity was counted using a gamma counter (Packard, Meriden, CT, USA). The percent specific lysis was calculated using the following formula: $100 \times$ [(experimental release−spontaneous release)/ (maximum release−spontaneous release)] for a mean of triplicate wells. Spontaneous release was obtained by target cells in the presence of medium alone and maximum release was obtained by the addition of 0·1 N HCl. Specific cytotoxicity is expressed in lytic units (LU). One LU was defined as the number of cells required for 30% specific target lysis (determined from dose-response curves with $r \geq 0{\cdot}95$), and results are expressed as number of LU per million PBMC.

Cell separation

Different cell populations were separated for determining the cell responsible for the lysis of K562 cells. Patients' PBMC were stimulated with Leishmania antigen (10 μg/ml) for four days in a humid atmosphere with 5% CO_2. These cells were separated using magnetic cell separation (Mini Macs, Miltenyi Biotec, Sunnyvale, CA, USA). The material was first magnetically stained using MACS microbeads (MoAbs conjugated to magnetic beads). Magnetic staining was done directly with specific MACS microbeads for $CD4^+$, $CD8^+$ subpopulations. In order to separate NK cells we used NK cell isolation kit, removing cells positive for CD3, CD4, CD19, and CD33. The stained material was passed through the Mini-Macs separator. Unstained material passed through the column, and retained cells were eluted after removing the column from the separator.

Statistical analysis

The curve for determining Lytic Units in each experiment was calculated from the dose-response determinations with 4 different effector:target cell ratios, by simple

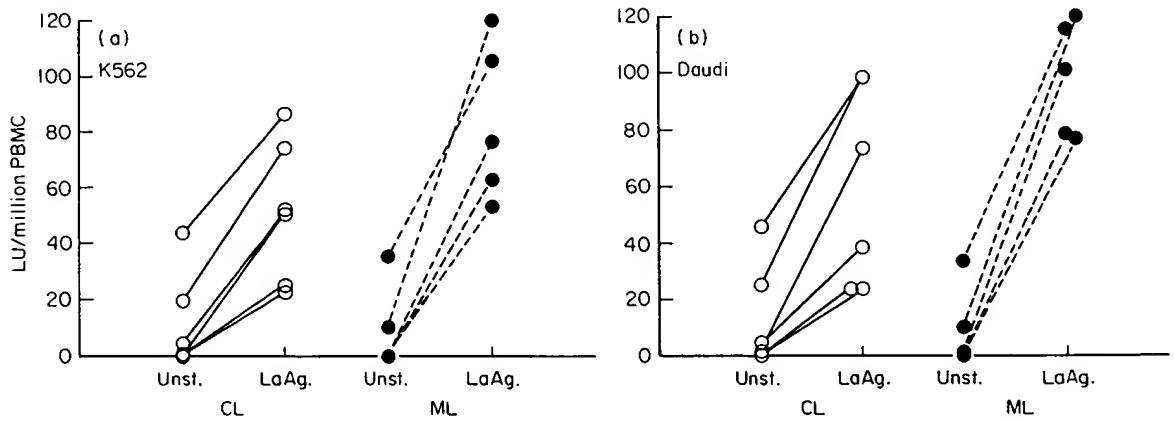

Figure 1 Effect of *L. amazonensis* antigen (LaAg; 10 μg/ml) on the cytotoxic effect of PBMC of cutaneous (CL) or mucosal (ML) leishmaniasis patients against K562 NK-sensitive tumour target cells (a) or Daudi NK-resistant tumour cells (b). Each point represent an individual patient with cells tested without stimulation (Unst.) or antigen-stimulated (LaAg.). Patient PBMC were incubated with LaAg. for four days, and then tested at different Effector:Target ratios at a ^{51}Cr-release cytotoxic assay. Results of specific lysis were used to calculate the number of 30% lytic units (LU) per million PMBC (see Material and Methods).

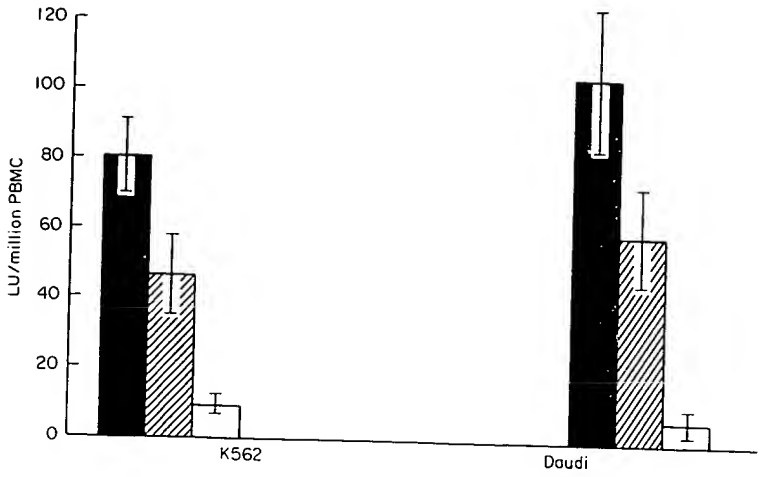

Figure 2 Comparison of mean cytotoxic responses of cutaneous (ribbed bars) or mucosal (solid bars) leishmaniasis patients with normal volunteers (open bars) PBMC against K562 or Daudi tumour target cells. Results represent the mean 30% lytic units (see Material and Methods) from Leishmania antigen-stimulated cells (10 μg/ml for four days) in all groups.

linear regression. Comparisons of the leishmania antigen-stimulated PBMC cytotoxic responses against Daudi and K562 target cells between CL and ML patients was performed by the Student's t-test. All tests were performed using the program GB-Stat version 4.0 (Dynamics Microsystems, Silver Spring, MD, USA).

RESULTS

Cytotoxicity against tumour cells

Figure 1 (left panel) shows that *L. amazonensis*-antigen was able to stimulate PBMC from cutaneous (CL) and mucosal (ML) leishmaniasis patients to lyse K562 tumour cells. Enhancement of cytotoxicity was observed in all tested patients. A similar pattern but with higher intensity was also observed against Daudi cells (Figure 1; right panel). Normal control PBMC ($n = 5$) stimulated with *L. amazonensis* antigen had 6·32 ± 3·72 LU/million cells against Daudi cells and 9·06 ± 2·78 LU/million cells against K562 target cells; much less than either CL or ML patients (Figure 2). Differences between CL and ML responses were not statistically significant (against K562 cells $t = 1·91$; $P = 0·11$; and against Daudi cells $t = 1·62$; $P = 0·15$). Unrelated proteins incubated at similar concentrations with patient PBMC did not promote increase in the cytotoxic response (data not shown). In order to determine the phenotype of the cells responsible for the cytolytic activity cell separation was performed. Figure 3 shows that the population enriched for NK cells retained almost all the lytic capacity, similar to the response of the unseparated cells, whereas $CD4^+$ and $CD8^+$ cells were not able to lyse this target. This kind of result was obtained in three different experiments, in both CL and ML patients (data not shown).

Cytokine regulation of cytotoxic responses

Antigen-induced cytotoxicity against K562 tumour target cells was modulated by both IL-10 and TGF-β. When *L. amazonensis* antigen-stimulated patient PBMC (10 μg/ml) were incubated in the presence of TGF-β (10 ng/ml), there was a marked reduction in cytotoxicity against K562 cells (Figure 4). A suppressive effect on the cytotoxicity assay was also observed when rHuIL-10 (40 U/ml) was added to leishmania antigen-stimulated

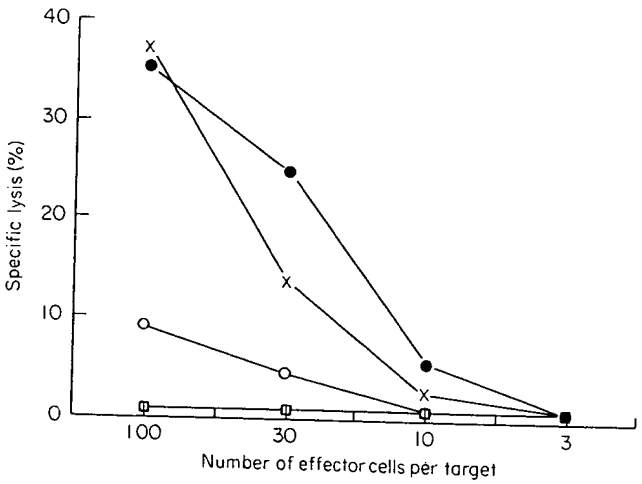

Figure 3 Determination of the phenotype of lytic cell population. PBMC were stimulated with leishmania antigen for four days, and then separated in $CD4^+$, $CD8^+$ and NK enriched populations by means of magnetic cell sorting. Lysis of ^{51}Cr-labelled K562 tumour target cells obtained with the unfractionated cells were compared to lysis observed in the presence of different cell populations. Results represent percent specific lysis at different effector-target ratios in one out of three similar experiments. ● total; □ $CD4^+$; ○ $CD8^+$; × NK.

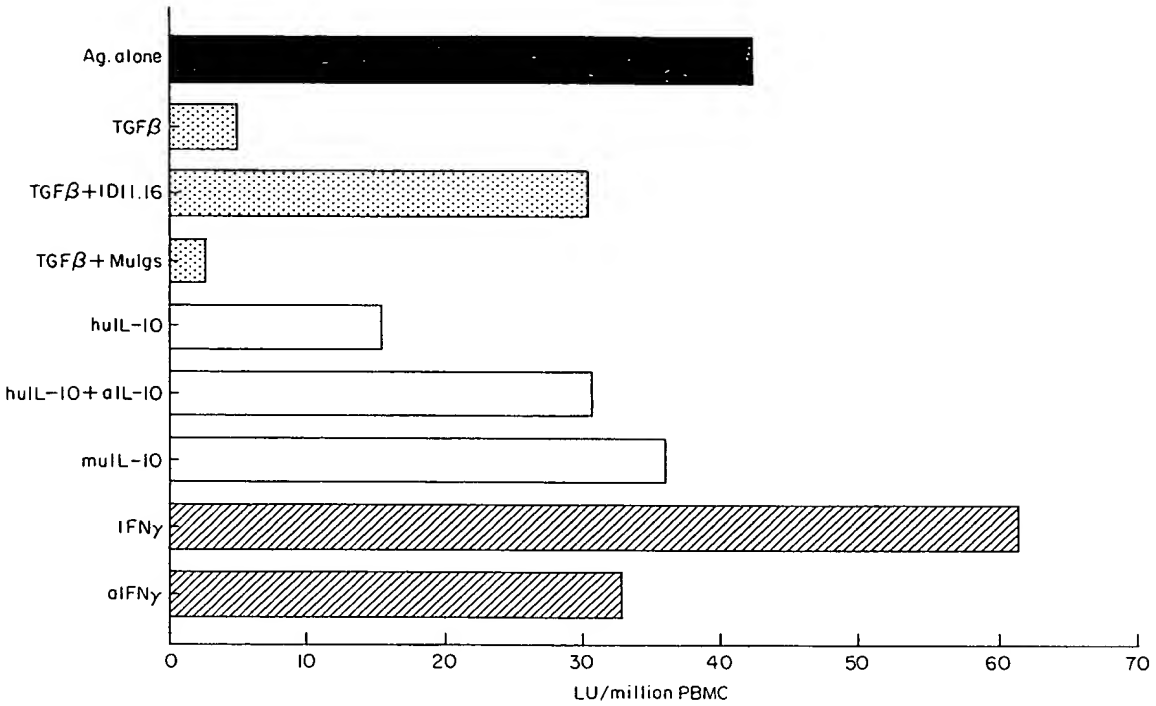

Figure 4 Effect of TGF-β, IL-10 and IFN-γ and its neutralizing antibodies on the cytotoxic effect of Leishmania antigen-pulsed PBMC from a CL patient against K562 tumour target cell. All cells were incubated with antigen (10 μg/ml) plus one of the following materials: the TGF-β (10 ng/ml); TGF-β (10 ng/ml) + 1D11.16 (10 μg/ml); IL-10 (40 U/ml); IL-10 (40 U/ml) + anti-IL-10 (10 μg/ml); IFN-γ (10 μg/ml); IFN-γ (10 μg/ml) + anti-IFN-γ (10 μg/ml). Incubations proceeded for four days and then the cells were used for the ^{51}Cr-released cytotoxic assay. When both the cytokine and its neutralizing antibody were used they were mixed together and incubated for 30 min previous to the addition to culture. Results presented in 30% lytic units/million PBMC. Representative experiment of four similar performed with cells from different donors.

PBMC against K562 cells (Figure 4). IFN-γ (10 μg/ml) by contrast led to increased cytotoxic response against K562 cells (Figure 4).

The suppressive effect of TGF-β was abrogated by the addition to culture of a mixture of pre-incubated TGF-β (10 ng/ml) and 1D11.16 (10 μg/ml), an anti-TGF-β MoAb (Figure 4). Similarly, pre-incubation of anti-IL-10 (10 μg/ml) was able to revert the suppressive effect induced by IL-10. No effect was obtained with murine recombinant IL-10 (Figure 4). The use of anti-IFN-γ (10 μg/ml) abolished the enhancing effect of IFN-γ on cytotoxicity of PBMC against K562 (Figure 4).

The suppressive effect of TGβ exhibited a dose-response fashion. Figure 5 shows the effect of different doses of TGF-β added to cultures of PBMC stimulated with *L. amazonensis*-antigen in two different patients' cells tested against K562 target cells.

Cytotoxicity against autologous cells

Cytotoxicity was also observed when antigen-stimulated PBMC were tested against *L. amazonensis* antigen-pulsed autologous macrophages. Table 1 shows that antigen induced increased cytotoxicity in all three patients tested; unstimulated PBMC gave negligible responses. Cytotoxicity against uninfected autologous cells was not observed. Furthermore IL-10 and TGF-β were able to depress the cytotoxic response against autologous targets (Table 1) similar to the effect observed with tumour target cells.

DISCUSSION

There are indications *in vitro* for the activity of lytic cells against macrophages parasitized by Leishmania (Pham & Mauel 1987), although such lysis may not result in parasite damage (Smith, Rodrigues & Russell 1991). Destruction of parasitized macrophages is observed *in vivo* following infection by *L. amazonensis* of immunized BALB/c mice, but rarely in naive animals of the same strain (Pompeu *et al.* 1992). NK cell activity had been reported in visceral leishmaniasis patients (Harms *et al.* 1991) and in mice infected by *L. donovani* (Kirkpatrick & Farrell 1984). In the present study we

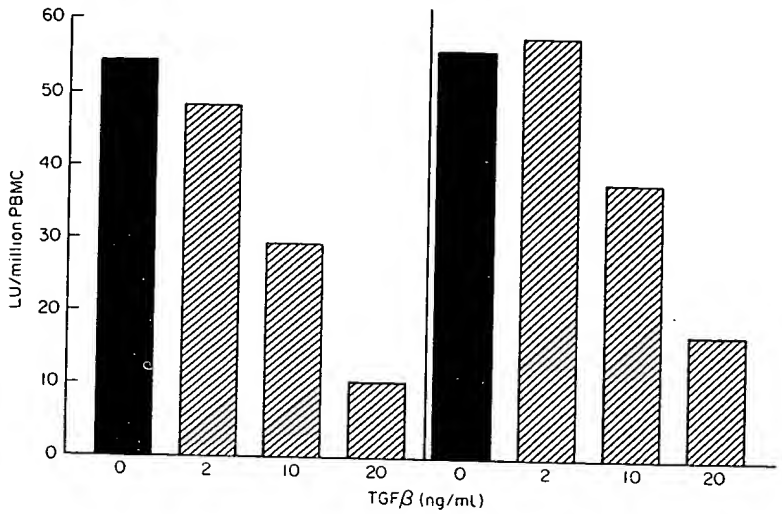

Figure 5 Dose response effect of TGF-β on the cytotoxic response of two mucosal leishmaniasis patients PBMC against K562 tumour target cells. PBMC were incubated with leishmania antigen (10 μg/ml) plus IFN-γ (20 IU/ml) without (solid bars) or with different doses of TGF-β (ribbed bars) for four days and used in the ^{51}Cr-released cytotoxic assay. Results of specific lysis were used to calculate the number of 30% lytic units (LU) per million PBMC (see Material and Methods).

show that lytic NK cells are also present in patients with tegumentary leishmaniasis, both in CL and ML patients.

Lytic cells have been associated with protection against leishmanial infection in the animal models in cutaneous leishmaniasis (Farrell, Muller & Louis 1989, Hill, Awwad & North 1989), but the role of cytotoxic cells in human disease has not been previously established. Cytotoxic phenomena may be involved in protection, lysing infected cells and contributing for reduction of parasite load, but may also be implicated in tissue destruction being deleterious to the host. It is important then to evaluate the presence and role of cytotoxicity in human leishmaniasis. The present study provides clear evidence for the existence of cytotoxic NK cells in the peripheral blood of tegumentary leishmaniasis patients which are stimulated by *L. amazonensis* antigen and capable of lysing K562 tumour cells.

Effects of cytokines on the immune mechanisms involved in leishmaniasis are essential in determining host and parasite protective responses. Th2 type cytokines such as IL-10 predominate in susceptible animals infected with *L. major* (Heinzel *et al.* 1991) and correlate with severity in human leishmaniasis (Ghalib *et al.* 1993).

IL-10 acts by depressing both tumour targets and autologous macrophage cytotoxicity in cutaneous leishmaniasis patients in this study. Since IL-10 is responsible for decreased antigen presentation by macrophages and inhibition of cytokine production by Th1 cells (Fiorentino *et al.* 1991) and macrophages (Fiorentino *et al.* 1991), this effect may be responsible for its inhibitory action in this system. In contrast, IL-10 has been shown to be a cytotoxic T cell differentiation factor in mice (Chen & Zlotnik 1991).

Another cytokine shown to be involved in the initial steps of leishmanial infection is TGF-β. It is produced by infected macrophages and leads to aggravated disease in the murine host (Barral-Netto *et al.* 1992). TGF-β is also responsible for altering the preferential Th1 to Th2 predominance in resistant animals (Barral *et al.* 1993) and has been shown to be produced within human lesions (Melby *et al.* 1994). It is possible that the IL-2 and IFN-γ produced by PBMC following antigen stimulation (Carvalho *et al.* 1985) are responsible for the enhanced activity of lytic cells observed *in vitro*. TGF-β inhibits the *in vitro* generation of cytotoxic T cells (Ranges *et al.* 1987, Mule *et al.* 1988) and NK cell activity (Rook *et al.* 1986), and this may be due to the blunting of IFN-γ action (Rook *et al.* 1986). It is not unexpected that TGF-β leads to depressed cytotoxic activity against autologous antigen-pulsed macrophages as observed in this study.

Cutaneous and mucosal leishmaniasis differ clinically, pathologically and may have diverse pathogenesis. No differences in cytotoxic activity *in vitro* have been

Table 1 Cytotoxicity against autologous antigen-pulsed macrophages in patients with tegumentary leishmaniasis

	Stimuli			
Patients' id	Antigen	Ag + IL-10	Ag + TGF-β	None
Antonio	35·7*	18·52	0·83	0·20
LTB 300	6·7	2·94	2·56	0·38
Carlito	41·7	ND	ND	0·91

* All results are expressed in lytic units/10^6 PBMC.

observed between these two disease presentations. It is possible that cytotoxic activity differ *in vivo* under the influence of diverse stimuli provided by in the lesion. In this regard it is important to point out that the mRNA for IL-10 is more abundant in ML lesions, (Cáceres-Dittmar *et al.* 1993) and the mRNA for IL-10 and TGF-β is increased in lesions with more than four months of duration, in comparison to early lesions (Melby *et al.* 1994). Since both cytokines are able to modulate cytotoxic activity it reinforces the possibility of *in vivo* differences in cytotoxicity between CL and ML patients. It is also important to point out that the present report deals primarily with cytotoxicity against tumour target cells and against autologous macrophages pulsed with parasite antigen. Such results cannot be extrapolated to Class I-mediated responses against infected macrophages, where CL and ML patients may differ in cytotoxic activity. Studies are under way in our laboratory to evaluate patients cytotoxic response to autologous Leishmania-infected macrophages.

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